Manipulating Somatic Cells to Remove Barriers in Induced Pluripotent Stem Cell Reprogramming

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A dissertation presented

by

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to

The Department of Molecular and Cellular Biology

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Biochemistry

Harvard University

Cambridge, Massachusetts

May, 2013

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in Induced Pluripotent Stem Cell Reprogramming

Abstract

Development leads unidirectionally towards a more restricted cell fate that is usually stable. However, it has been proven that developmental systems are reversible by the success of animal cloning of a differentiated somatic genome through somatic cell nuclear transfer (SCNT). Recently, reprogramming of somatic cells to a pluripotent embryonic stem cell (ESC)-like state by introducing defined transcripton factor has been achieved, resulting in the generation of induced pluripotent stem cells (iPSCs), which resemble ESCs. iPSC reprogramming is of great medical interest, as it has the potential to generate a source of patient-specific cells. However, the dangerous delivery method, low efficiency, and slow kinetics of the reprogramming process have hampered progress with this technology.

To tackle these blockades of reprogramming and generate clinically useful iPSCs, we have modulated normal developmental signal transduction pathways of keratinocytes, namely Notch signaling using a chemical. Notch inhibition by a chemical inhibitor, DAPT, improves the efficiency of reprogramming keratinocytes to pluripotency. Inhibition of Notch replaces two of the four exogenous reprogramming factors, oncogene *Klf4* and *cMyc*. Importantly, pharmacological inhibition of Notch provides an

escape from suppression of p53 activity, which is required to enhance reprogramming without *Klf4* and *cMyc* and possibly leads to genetic mutations.

In another approach to improve the efficiency and kinetics of reprogramming, we have generated an allelic series of DNA methyltransferase1 (*Dnmt1*) mouse embryonic fibroblasts (MEFs) and reprogrammed them with four reprogramming factors. A cell line that has 90% reduced *Dnmt1* levels decreases the methylation levels of *Oct4* regulatory regions. Significantly, the hypomorphic *Dnmt1* cell line enhances the efficiency and dynamics of reprogramming by directly activating Oct4 and bypassing the intermediate states.

Ultimately, the knowledge gained from experimental manipulation of cell fates and epigenetic barriers in iPSC reprogramming will contribute to a better understanding of the reprogramming technology and its controlled mechanism to achieve pluripotency. Furthermore, our study hopes to facilitate the development of clinical applications to personalized regenerative medicine.

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List of Abbreviations

ALS	Amyotrophic lateral sclerosis
AP	Alkaline phosphates
AZA	5-azacytidine
BMP	Bone morphogenic protein
ChIP	Chromatin- immunoprecipitated
CpG	Cytosine-phosphate-guanine
Dnmt1	DNA methyltransferase1
Dnmt3a (or 3b)	DNA methyltransferase3a (or 3b)
EBs	Embryonic Bodies
EGF	Epidermal growth factor
ES (or ESCs)	Embryonic Stem (Cells)
FACS	Fluorescence-activated cell sorting
Fbx15	F-box contiaining protein 15
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
GSK3β	Glycogen Synthase Kinase 3β
H3K4me3	Histone H3 lysine 4 trimethylation
H3K27me3	Histone H3 lysine 27 trimethylation
HGF	Hepatocyte growth factor
HPV	Human papillomavirus
IAP	Intracisternal a particle
ICM	Inner cell mass
iDOT1L	Inhibitor of the histone methyltransferase DOT1L
iMN	Induced motor neuron
iN	Induced neuron
iPS (or iPSCs)	Induced pluripotent stem (cells)
KSCs	Keratinocyte stem cells
LIF	Leukemia inhibitory factor
MEFs	Mouse Embryonic Fibroblasts
miRNA	microRNAs
NSCLC	Non–small cell lung carcinoma
NSCs	Neural stem cells
NPCs	Neural progenitor cells
mESCs	Mouse embryonic stem cells
mRNA	Messenger RNA
PcG	Polycomb group
PRC1 (or 2)	Polycomb-repressive complex 1 (or 2)
PCR	Polymerase chain reaction
PDK1	3’-phosphoinositide-dependent kinase-1
qPCR	Quantitative polymerase chain reaction

RA	Retinoic acid
RT-PCR	Real-time polymerase chain reaction, or reverse transcriptionpolymerase chain reaction
SHH	Sonic Hedgehog
SMA	Spinal muscular atrophy
SMN	Survival of motor neuron
SSEA-1	Stage specific embryonic antigen-1
SV40T	Simian virus 40 large T
T-ALL	T-cell acute lymphoblastic leukaemia
TCRβ	T-cell-receptor-β
TGFβ	Transforming growth factor-β
TrxG	Trithorax
VPA	Valproic acid
WD5	WD repeat domain 5

Acknowledgements

I would like to first thank my advisor, professor Kevin Eggan, who has given me indispensable guidance and kept science very exciting to me. He always motivated and help me to overcome many challenges. Special thanks to my dissertation advisory committee, professor Gregory Verdine, professor Briana Burton, and professor Alexander Meissner for advice and encouragement over the years.

I appreciate all the help from Dr. Justin Ichida who was a former postdoctoral fellow with Eggan lab which I've had the opportunity to work alongside and collaborate with. The keratinocyte reprogramming studies in Chapter 2 were done with Dr. Justin Ichida. The Dnmt1 genome-wide investigations in Chapter 3 were done in collaboration with professor Alexander Meissner, Camille Sindhu, Jack Smith, and Rahul Karnik. By professor Lee Rubin's support, Anthony Arvanites and Vatsal Oza worked with me on several chemical screenings. I would deeply like to thank all for their insights, contributions, scientific visions, and interesting findings in research.

I enjoyed the help from many of my colleagues in Eggan lab who are hard-working and intelligent people. Special thanks to Jack Sandoe, who always made time to edit my papers. It was great to talk with Atsushi Into, Florian Merkle, and Evangelos Kiskinis, who I have discussed many challenging experiments with. Thanks to Joe Klim, Brandi Davis-Dusenbery, Seun Jonson-Akeju, Ava Carter, Shila Mekhoubad, Sophie de Boer, Luis Williams, Steve Han, Katie Koszka, Nick Atwater, Diane Santos, and former members, Mariko Yamaki, Gabriella Boulting, Inna Tabansky, Esther Son.

I would also like to acknowledge my classmates in the Department of Molecular and Cellular Biology here at Harvard University. Especially my first year, it was a pleasure

discussing and solving many problems with my classmate André Green. For those names that I have not mentioned, thank you all for your time and support.

Finally, I have many thanks to my fiancé, Mr. Tony TCW, who woke me up from the first day we met, when I was having such a hard time in my life. He supported me every day in every angle. He continued to inspire me to focus and stay positive, even though he saw and heard so much negativity around me. He said "Your prayers and ambitions will help you to see the finish line." I am very excited to celebrate this long hard journey with him.

Thank you!

May 2013

Cambridge, MA

Chapter 1

The Progress of Induced Pluripotent Stem Cell Reprogramming

Abstract

Ground-breaking advances in nuclear reprogramming have been made by reversing the normal development using defined factors. A small set of transcription factors (*Sox2, Oct4, Klf4* and *c-Myc*) directly confer embryonic stem cell (ESC)-like characteristics to somatic fibroblasts. The resulting induced pluripotent stem cells (iPSCs) are self-renewing and pluripotent. However, the low efficiency and viral delivery of transgenes provide a significant handicap for clinical applications as well as mechanistic studies. Therefore, many research groups have proposed various approaches to tackle the technical limitations and understand the systemic challenges to induce pluripotency. Moreover, iPSCs can teach us about principles of normal development and disease, which may ultimately apply to patients for custom-made cell therapy.

Introduction

Animal development starts as the fertilized egg undergoing a programmed process of cell proliferation and differentiation that generates the wide variety of cell types which an individual is composed of. Decades of research in cell fate changes during development have led to the view that this differentiation process, *in vivo*, is irreversible, and differentiated cells are committed to their cell fate. However, pioneering studies from somatic cell reprogramming with *Xenopus levies* (Gurdon et al., 1958) to the cloning of Dolly the sheep proved that fully differentiated somatic cell nuclei can be reprogrammed back to an embryonic-like state by factors present in oocytes (Wilmut et al., 1997). The recent breakthrough made by Yamanaka and colleagues revealed that differentiated somatic cells can reverse back to a pluripotent state *in vitro* by the introduction of a defined combination of transcription factors that are highly enriched in embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006).

The beauty of this powerful technique, transcription-factor-induced reprogramming to ESC-like states, lies in its simplicity and robustness. Many different cell types from the wide range of species including human can also be reprogrammed to pluripotency by ectopic over-expression of certain factors, raising the possibility of the clinical application of personalized stem cell-based therapies without immune rejection or ethical concerns as well as studying genetic diseases *in vitro*.

This opening chapter focuses on the development of pluripotency reprogramming technology and various approaches to overcome its limitations to produce safer and applicable stem cells that could directly differentiate into target cell

types for cell replacement therapies. The following body of work clearly demonstrates the possibility of manipulating cell fate to pluripotency by new ways and of lowering the epigenetic barrier to achieve pluripotency.

State of the Art Technology in Stem Cells

The Pluripotent Stem Cell

The years since Takahashi and Yamanaka's breakthrough have seen the term pluripotency assigned to a variety of cell types with a wide range of functional capacities. To understand their discovery, we first need to define what it means to be pluripotent.

The term pluripotency describes a cell that has the ability to differentiate into cell derivatives of all three primary embryonic germ layers: the ectoderm, mesoderm, and endoderm. More stringently, it describes a cell that can give rise to an entire organism, generating every cell type in the organism. The property of cell pluripotency was first discovered, when two cells of an early sea urchin blastocyst that were separated generated two complete sea urchins (Driesch, 1891). Many decades later, research of embryo aggregation and blastocyst chimaerism in the 1960s and 1970s solidified the idea that the cells of the inner cell mass (ICM) of the mouse blastocyst were pluripotent (Brinster, 1974; Dewey et al., 1977; Gardner, 1968).

This powerful developmental potency, pluripotency, which gives rise to form any cell type of interest, brings attention to the usefulness in a clinical and scientific standpoint. For this purpose, an ideal cell type, embryonic stem cells (ESCs) were derived *in vitro* from the ICM of the pre-implantation embryo, possessing the ability to self-renew and the capacity to generate definitive ectoderm, mesoderm, and endoderm-derived lineages. The first derivation of ESCs was performed from mouse blastocystes in 1981 (Evans and Kaufman, 1981; Martin, 1981), leading to the era of culturing

pluripotent stem cells in a dish. Then the first successful human ESCs derivation was followed in 1998 (Thomson et al., 1998), opening a door to the idea of cell therapy.

Due to the pluripotent and self-renewing nature of ESCs, there has been an interest to convert adult somatic cells into ESCs, named nuclear reprogramming. The oldest reprogramming method is somatic cell nuclear transfer (SCNT)-the genetic material from the donor cell being reprogrammed is transferred to an enucleated oocyte from the recipient that has reprogramming activity, resulting in the development of a new organism (Gurdon and Byrne, 2003). To generate patient-specific ESCs, there have been considerable efforts to clone blastocystes from patient somatic cells by SCNT. However, SCNT carries not only ethical concerns regarding the destruction of human embryos, but also the shortage of donated eggs for the use of recipient cells in nuclear transfer. Moreover, due to technical difficulties, it is a challenge to achieve successful SCNT in human (Egli et al., 2011a).

Next, another nuclear reprogramming method, called cell fusion, had been executed by fusing pluripotent ESCs and differentiated cells, generating a pluripotent heterokeryon. Tada and colleagues established the ES-thymocyte hybrid cells by fusing adult thymocytes with ESCs, which contribute to all three primary germ layers of chimeric embryos (Tada et al., 2001). Likewise, Eggan and collegues produced human somatic hybrid cells by fusing human BJ fibroblasts with a human ESC line (Cowan et al., 2005). The resulting cells have morphology, marker expression patterns, and differentiation potential characteristic of human ESCs. Although cell fusion is useful for

studying reprogramming, it has a limitation in therapies due to the tetraploid DNA content of the hybrids.

Induced Pluripotent Stem Cells (iPSCs)

Despite many hindrances to derive human ESCs, researchers have explored the pathways which derive pluripotency and regulate the maintenance of ESCs. In 2006 Takahashi and Yamanaka developed a groundbreaking technology in the field of nuclear reprogramming. They found that differentiated somatic cells can be reprogrammed to a pluripotent state by a set of defined factors (Takahashi and Yamanaka, 2006). The resulting cells are named induced pluripotent stem cells (iPSCs), which could be an alternative source of personalized patient-specific stem cells.

Previously, there have been reports of factor-mediated cell conversion from one cell type to another within closely related lineages that share developmental history. Discovering a key component such as a specific transcription factor or environmental products that distinguish different cell types within the same lineage during differentiation allowed cell conversion from fibroblasts to myoblasts in the mesodermal lineage (Davis et al, 1987), from B cells to macrophages within the ectodermal origin or the hematopoietic system (Kulessa et al., 1995; Nerlov and Graf, 1998; Xie et al., 2004), or from pancreatic cells to liver cells between the endodermal lineages. In developmental perspective, Takahashi and Yamanaka's finding is a historic contribution because reprogramming somatic cells to pluripotency is a complete reversal of normal developmental processes just by a few defined factors. Interestingly, Takahashi and

Yamanaka's finding provides a lesson that differentiated cells can be genetically engineered to achieve pluripotency rather than the necessity of the complex environmental factors such as the cytoplasm of an egg or an ESC.

By screening 24 candidate genes selected for their links to ESC pluripotency, they found four reprogramming factors—Oct4, Sox2, Klf4, and cMyc—that are sufficient to directly confer ESC characteristics to somatic fibroblasts (Takahashi and Yamanaka, 2006 and 2007). They reprogrammed mouse embryonic fibroblasts (MEFs) and adult fibroblasts to pluripotent ES-like cells by over-expressing, by retrovirus-mediated transduction, four transcription factors followed by selection for activation of the Oct4 target gene, first-generation F-box containing protein 15 (*Fbx15*). Rarely observed ES-like colonies that had activated *Fbx15* were called iPSCs.

These Fbx15-positive iPSCs demonstrated pluripotency by their ability to form teratoma. However, they failed to contribute to adult chimeras. Although this pluripotent state depended on the viral over-expression of *Oct4* and *Sox2* genes, the core ESC factor, such as endogenous *Oct4* and *Nanog* genes were either not expressed or expressed at a lower level than in ESCs. Also, they showed incomplete demethylation of the promoters of *Oct4* and *Nanog*. Therefore, the *Fbx15*-activated iPSCs did not correspond to ESCs, implying that they represented an incomplete state of reprogramming. By using the *Oct4* or *Nanog* selection method, which is a more stringent selection criterion for pluripotnecy, the *Oct4* or *Nanog*-activated iPSCs were fully reprogrammed iPSCs. Their global gene expression profiles were indistinguishable from those of ESCs. In contrast to the *Fbx15*-iPSCs, they were hypomethylated in endogenous *Oct4* and *Nanog* promoters. Also, they

generated the wide range of tissues in a chimera and contributed to the germ line (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Importantly, they generated last gestation embryos through tetraploid complementation, the most stringent test for developmental potentcy (Wernig et al, 2007). Therefore, these iPSCs exhibited all the molecular and functional characterstics of ESCs, indicating that Oct4 and Nanog positive iPSCs were indistinguishable from ESCs.

In 2007, Yamanaka and Thomson's group independently announced the successful derivation of iPSC from human fibroblasts (Takahashi et al., 2007; Yu et al., 2007). The resulting human iPSCs closely resemble human ESCs in their morphology, gene expression and the epigenetic status of pluripotency genes, and can differentiate into the cells of the three germ layers *in vitro* and *in vivo*. The derivation of human iPSCs holds enormous potential for regenerative medicine.

Technical Limitations of iPSC Reprogramming

Although iPSC technology is a promising resource in regenerative medicine, the clinical utility of this technique is compromised by two main problems. First is the delivery method to generate iPSCs; the retrovirus-mediated transgenes tend to induce malignant transformation. Mice derived from iPSCs frequenctly developed cancer (Okita et al., 2007). Secondly, the efficiency of reprogramming is extremely low (~0.001-1%) (Takahashi and Yamanaka, 2006).

In addition to considering the induction of malignant transformation by the retroviral delivery of the reprogramming factors, it was questioned whether insertional

mutagenesis could potentially be required for iPSC reprogramming. Previous reports have shown that replication-defective retroviral integrations themselves were able to activate endogenous genes to promote survival in hematopoietic stem cells *in vitro* (Kustikova et al. 2005). Similarly, we have to contemplate the possibility that one or several copies of the virus in iPSCs might integrate into and activate a gene that triggers the acquisition of a pluripotent state. When iPSCs were derived from fibroblasts using retroviruses, they carried ~10- 20 proviral transgenes that expressed *Oct4, Sox2, Klf4* and *cMyc*, which were found at different copy numbers per clone (Maherali et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). In 2008, Yamanaka and colleagues generated iPSCs from lineage-committed differentiated epithelia cells, primary hepatocytes (liver cells) and gastric epithelia cells (stomach cells) using the same reporter and the selection system they used in the first generation of the fibroblast-derived iPSCs (Aoi et al., 2008). They showed that iPSCs derived from hepatocytes and stomach cells had fewer viral integration sites than those in fibroblast-derived iPSCs. In addition, the sequencing result of viral insertion sites in iPSCs derived from fibroblasts, stomach cells, and liver cells did not show any common integration sites, suggesting that insertional mutagenesis does not contribute to the induction of pluripotency (Aoi et al., 2008; Varas et al., 2008). Therefore, the transgenic methods of iPSC generation that could potentially cause cancer needed to be improved.

It is conceivable that the frequency at which a single somatic cell gets infected by the four viral transgenes at the same time in the appropriate stoichiometry is extremely low, resulting in the low overall efficiency of reprogramming. To address the

question of whether viral infection is indeed the rate-limiting step, several groups have generated a 'secondary system,' in which cells can reactivate all four factors at the correct stoichiometry by using a doxycycline-inducible system, assuming that it should give rise to iPS cells at an efficiency close to 100% based on the theory that nearly100% of the four reprogramming factors can be reactivated (Hockemeyer et al., 2008; Maherali et al., 2008; Wernig et al., 2008a). When secondary mouse or human fibroblasts are treated with doxycycline, 3-5% of the cells gave rise to iPSCs, that is, a 30- to 100-fold increase in efficiency compared to primary fibroblasts infected directly with viruses, indicating that viral infection and expression can affect the efficiency of reprogramming. However, the frequency of iPSC generation is still unexpectedly low, suggesting that the expression of the four factors alone is insufficient in itself to reprogram somatic cells to pluripotency and that there is a roadblock such as epigenetic barriers that must affect the overall efficiency of reprogramming.

Tackling Obstacles of iPSC Reprogramming

Toward Transgene-free iPSC Reprogramming

A variety of technologies have been developed to generate transgene-free iPSCs for clinical practice. iPSCs are initially produced by using retroviral or lentiviral transduction of transcription factors, and these transgenes are randomly inserted into the host genome, holding a risk of tumorgenicity. In fact, chimeras made with iPSCs harboring the most potent proto-oncogene *cMyc* have a high incident of tumor formation

due to the reactivation of the *cMyc* transgene expression (Okita et al., 2007). First, in an effort to reduce this risk, it has been shown that iPSCs can be derived in the absence of the *cMyc*, albeit with a low efficiency of reprogramming (Nakagawa et al., 2008; Wernig et al., 2008).

Several alternative methods for delivering these reprogramming factors other than through retrovirus or lentivirus have been developed, so called non-integrative or excisable virus approaches for iPSC derivation. Researchers have established iPSCs by using adenovirus or transient plasmids, which are non-integrating (Okita et al., 2008; Stadtfeld et al., 2008b), and transposon or loxP-flanked lentiviral, which are removable (Somers et al., 2010; Woltjec et al., 2009). Moreover, mouse and human iPSCs could be generated by the direct delivery of recombinant reprogramming protein (Kim et al., 2009a; Zhou et al., 2009). The overall efficiency of these reprogramming technologies was one or two magnitudes lower than the rates of retrovirus or lentivirus methods. Thus, the original retrovirus delivery method for the generation of iPSCs has been improved by avoiding viral insertions into the iPSCs genome, however, all of these trials still could not overcome the low efficiency of reprogramming.

Due to the low frequency of producing iPSCs with these transient expression methods, researches have been directed toward increasing the efficiency by seeking further optimizations for future research or therapeutics. Many efforts have been made to improve the reprogramming efficiency as well as avoid transgene integration risk by searching for chemicals or signaling pathways that may govern the mechanism of reprogramming. Chromatin modifiers, various growth factors, and many other chemical

compounds have been reported which could not only improve the efficiency of reprogramming but also be used for reprogramming transgene substitutes. Histone deacetylase inhibitors, for example, valproic acid (VPA) improves the reprogramming efficiency by more than 100-fold and also allows reprogramming without *cMyc* in fibroblasts (Hungfu et al., 2008a). Following studies have shown that VPA enabled reprogramming human fibroblasts into iPSCs with only two factors, *Oct4* and *Sox2* (Hungfu et al., 2008b). Histone methyltransferase inhibitor, BIX-01294, significantly increases the efficiency of reprogramming and can substitute for *Sox2* in MEFs (Shi et al., 2008a and 2008b). In addition, small molecule inhibitors that control signaling pathways, such as Wnt 3A, ALK5 inhibitors, and Tgf- β inhibitors have also been found to improve the induction of iPSC generation and replace reprogramming factors (Hungfu et al., 2008a; Ichida et al., 2009; Maherali et al. 2009; Shi et al., 2008b). For example, RepSox, which is a functional target of Tgf-β type I receptors, specifically ALK4/5 as well as ALK2, is an efficient small molecule replacement of *Sox2* and *cMyc* and facilitates iPSC reprogramming (Ichida et al., 2009). Vitamin C treatment or exposure to a hypoxic environment has also found to enhance the generation of iPSCs (Esteban et al., 2010).

Along with chemical approaches, the tumor suppressor protein p53 and cell cycle regulator INK4A have been reported to act as a barrier to the reprogramming of somatic cells to iPSCs. The repression of these genes removes cell-cycle control checkpoints, thereby increasing reprogramming efficiency (Kawamura et al., 2009; Marión et al., 2009; Li et al., 2009; Utikal et al.,2009). The combination of *p53*siRNA and a

transcription factor, UTF1, with the reprogramming factors increase the efficiency of iPSC generation from human embryonic fibroblasts more than 100-fold (Zhao et al., 2008). Another transcription factor, ESRRB, has also been found to enhance the reprogramming efficiency and replace *Klf4* (Feng et al., 2009). However, none of these chemical compounds, growth factors, or signaling control can generate transgene-free iPSCs.

Recently, iPSCs have been generated from mouse and human somatic cells by using direct transfections of mature microRNAs (miRNA) (Miyoshi et al., 2011), which are well-characterized regulators of development and differentiation (Lee et al., 1993; Ruvkun, 2001). The miRNAs techniques have benefits from the perspective of potential clinical translation due to the fact that this reprogramming method does not require vector-based gene transfer. It also shows efficient and faster reprogramming kinetics than retroviral or lentiviral vectors, however it does operate at considerably lower efficiency.

More recently, iPSC were derived from fibroblasts using modified RNA encoding the iPS reprogramming factors, resulting in transgene-free iPSCs at higher efficiency (Mandal et al., 2013; Warren et al., 2010). The mRNAs were synthesized by using *in vitro* transcription reactions and delivered into the cytoplasm by endocytosis. In light of their study, they overcome cytotoxicity upon exogenous RNA transfection by modifying mRNAs, which are synthesized with the complete substitution of uridine and cytidine with the modified ribonucleotides pseudouridine and 5-methylcytidine, thereby allowing robust and sustained protein expressions of iPS factors. Modified mRNA

technology raises hope for cell-based therapeutic applications by large-scale production of transgene-free, patient specific iPSCs. However, it still retains some disadvantages. It is costly, labor intensive, and technically challenging, since this method requires a repeated administration of modified RNAs due to its labile nature and relatively short half-life. Also, it shows relatively slow kinetics. Thus, it will require further optimization to be useful in research or for future therapeutic purpose.

Starting with Various Somatic Cell Types

iPS technology has made major progress towards a transgene-free reprogramming, nevertheless the cell origins and molecular mechanisms of iPSC induction remain elusive. The low efficiency of the iPSC derivation has been argued to depend on the presence of rare stem cells within the starting population, because the frequency of the adult stem cells' existence in many tissues is about the same as the success rate of iPSC reprogramming. The Jaenisch group first attempted to reprogram terminally differentiated mature B lymphocytes to pluripotency to evaluate whether terminally differentiated cells can give rise to iPSCs (Hanna et al., 2008). B cells carry differentiation-associated DNA rearrangements, which serve as unequivocal genetic markers of their differentiation state (Hochedlinger and Jaenisch, 2002). The ectopic expression of *Oct4*, *Sox2*, *Klf4* and *cMyc* alone was not sufficient to reprogram B lymphocytes into iPSCs, even applying to 'secondary' systems (Wernig et al., 2008a), however, terminally differentiated adult cells could reprogram to iPSCs by either additional over-expression of the transcription factor Cebpα or knockdown of the

transcription factor Pax5, which maintained B cell identity (Hanna et al., 2008). The Hochedlinger group also proved that terminally differentiated pancreatic β cells that were genetically marked could be reprogrammed to iPSCs by four reprogramming factors, although with a low frequency (Stadtfeld et al., 2008a). These results demonstrated that adult stem cells were unlikely to be the selective cell type in successful reprogramming experiments.

Many groups then started to reprogram with a wide variety of differentiated cell types such as neural progenitors, melanocytes, and keratinocytes with an idea that different cell biology—gene expression profile, epigenetic status, developmental potential etc. —may significantly contribute to increasing reprogramming efficiency and possibly reducing iPSC factors. It is possible different somatic cell types have different endogenous gene expression profiles that may aid in their reprogramming. From the previous reports that iPSCs derived from fibroblasts using retroviruses have different copy numbers of each of the four factors, we can predict that precise relative amounts of the individual transcription factors are important for reprogramming (Maherali et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). This is consistent with observations that Oct4 and Sox2 levels in ESCs are critical for maintaining a self-renewing pluripotent state (Kopp et al., 2008; Niwa et al., 2000). Therefore, if each of different cell types expresses certain amounts of endogenous iPS genes or other pluripotency related genes, these levels of expressions in the particular cell type may allow the cell to achieve pluripotency without certain iPSC factor and/or facilitate reprogramming efficiency.

In addition, when iPSCs were derived from hepatocytes and stomach cells, the viral infectivity of these different cell types was different from those of fibroblasts. iPSCs were obtained with a lower efficiency of retrovial transduction (30 to 45%), although they could not be generated from MEFs at the same infection ratio (Aoi et al., 2008). This indicates that different cell types other than fibroblasts could increase the efficiency of reprogramming, due to a higher acceptance of viral transduction.

With respect to iPS factor reduction, one of the major advances has shown that neural stem cells (NSCs) transduced with only *Oct4* can be reprogrammed into iPSCs (Kim et al., 2009). Consistent with the notion that, the number of reprogramming factors can be reduced when certain somatic cell type endogenously expresses appropriate levels of complementing factors to induce pluripotency, NSCs express higher levels of endogenous *Sox2*, *cMyc*, and *Klf4* than those of ESCs, which makes them reprogram to iPSCs in the absence of these three reprogramming factors. When transduced with two factors, NSCs can dramatically increase the reprogramming efficiency; mouse NSCs can give rise to iPSCs up to 50 times more efficiently than mouse fibroblasts (Kim et al., 2008). In addition to the high expression levels of *Sox2*, *cMyc*, and *Klf4*, NSCs endogenously express several other pluripotency markers, such as SSEA-1. This may possibly make them reprogram faster and more efficient than MEFs. However, NSCs are an undesirable cell type due to their difficult accessibility, when considering future clinical application of iPSCs.

Several groups have evaluated whether melanocytes or keratinocytes from ectodermal origin which are easily accessible from patient's skin are also able to be

reprogrammed into iPSCs. Melanotyes did not require ectopic *Sox2* expression for the conversion into iPSCs due to their endogenous program and gave rise to iPSCs at three times higher efficiencies than fibroblasts (Utikal et al., 2009b). Moreover, by four iPS factor reprogramming, human keratinocytes underwent reprogramming at least 100- fold more efficient and two-fold faster than human fibroblasts (Aasen et al., 2008; Maherali et al., 2008) Keratinocytes can also be reprogrammed only with three factors (*OCT4*, *SOX2*, *KLF4*) due to their higher expression level of *CMYC* than that of fibroblasts (Aasen et al., 2008).

A pluripotent stem cell state has now been induced in a plethora of differentiated cell types: from mouse to human fibroblasts, and then to a wide variety of other cell types, including pancreatic β-cells, NSCs, mature B cells, stomach cells, liver cells, melanocytes, adipose stem cells, and keratinocytes, demonstrating the seemingly universal capacity to alter their cellular identity to iPSCs. However, the frequencies of converting these various cell types to a pluripotent state and the possibility of iPS factor replacements by the endogenous genetic profile vary, suggesting that the cell type selection affects efficient iPSC generation. Thus, the best combination of the right cell type to facilitate reprogramming and transgene-free methods can bring iPSC technology realistically closer to clinical application.

However, there still remained several unsolved questions. One of the main questions that needed to be investigated is the identity and property of the starting cells that give rise to iPSCs. NSCs were less differentiated progenitor states. Keratinocyte cultures contained more progenitors than in fibroblast cultures. It is possible that less

differentiated somatic cells can be reprogrammed more efficiently than more or terminally differentiated cells. Alternatively, keratinocytes have a transcriptional state (Dotto, 2008), which could be more amenable to reprogramming than fibroblasts. It will undoubtedly be interesting to investigate the question of whether the less differentiated state of a cell or certain cell property, such as self-renewing capacity, can affect its reprogramming efficiency into iPSCs.

Overcoming Epigenetic Barriers

Epigenetic Barriers

In 1957, Conrad Waddington proposed the concept of an epigenetic landscape, which represented the cellular decision-making process during development (Waddington, 1957). Epigenetics is a phenomenon that changes the final outcome of a locus or chromosome, resulting in establishing and maintaining stable cellular phenotypes, without changing the DNA sequence. For example, cellular differentiation could be considered an epigenetic phenomenon, because it generates diverse cell types with disparate gene expression profiles and distinct cellular functions, while sharing an identical genotype in a multicellular organism. During differentiation, cells achieve their own lineage by adopting distinct states that are highly resistant to perturbation. An important mechanism for lineage restriction involves epigenetic changes that are nonetheless stable and heritable (Goldberg, 2007).

The best characterized epigenetic machinery is DNA methylation, which is a stable and heritable mark involved in many biological processes, including gene regulation, X-chromosome inactivation, and genomic imprinting. It occurs on cytosine residues of CpG dinucleotides and correlates with transcriptional repression (Goll and Bestor, 2005). Tissue-specific patterns of DNA methylation are established by *de novo* DNA methyltransferase Dnmt3a and Dnmt3b and followed by Dnmt1, the enzyme that maintains DNA methylation patterns through mitosis in a somatic genome (Okano et al., 1999). *Dnmt1* knockout causes early embryonic lethality (Li et al., 1992), and conditional deletion of *Dnmt1* is not tolerated in somatic cell types (Jackson-Grusby et al., 2001), indicating the importance of maintaining global DNA methylation patterns in cellular differentiation and identity.

A variety of covalent modifications to histone proteins that compact DNA into nucleosomes is another important signature of cell identity (Wang et al., 2004). The N- and C-terminal tails of histone H3 are extensively methylated, acetylated, phosphorylated and ubiquinated in a complex manner reflective of the transcriptional status at the region. For example, pluripotent stem cells contain a characteristic chromatin signature, termed ‘bivalent domains’(Bernstein et al., 2006 and 2007) —that is, enriched for both activating histon H3 lysine 4 trimethylation (H3K4me3) and repressive histone H3 lysine 27 trimethylation (H3K27me3) modifications (Mikkelsen et al., 2007). First, Lender and colleagues identified a bivalent feature specific to ESC, while differentiated cells either had a univalent state or lost both marks, using chromatin-immunoprecipitation (ChIP) followed by hybridization to microarrays (ChIP-

Chip). Later, using ChIP-seq (chromatin-immunoprecipitated DNA followed by high-throughput sequencing technology), Mikkelsen and colleagues demonstrated that bivalent domains are indicative of genes that remain in a poised state. Although pluripotent cells contained high numbers (~2500) of bivalent domains, multipotent neural progenitor cells (NPCs) that were more differentiated than ESCs remained bivalent (~200), indicating that they still retain the lineage of choice (Meissner et al., 2008). These results are consistent with the involvement of histone epigenetic modifications in determining cell fate.

Overcoming Epigenetic roadblocks

For a detailed description of the epigenetic status of cells at different stage of development in Waddington's epigenetic landscape model, totipotent zygotes have global DNA demethylation. Pluripotent cells, such as ESCs or iPSCs, have in general hypomethylated promoter and global repression of differentiation genes by Polycomb proteins, whereas differentiated cells have hypermethylated promoters and de-repressed lineage genes (Waddington, 1957). The epigenetic status of differentiated somatic cells and pluripotent stem cells is largely different. Therefore, reprogramming differentiated somatic cells to pluripotency may provide active mechanisms by which epigenetic modifications are reversed, although the barrier is formidable.

To achieve the pluripotent state from somatic cells, extensive remodeling of epigenetic marks that control access to genes and regulatory elements in the genome are essential. The re-establishment of H3K4me3 and the loss of DNA methylation in 'ES-

cell-associated transcript' (ECAT) genes, such as *Oct4* and *Nanog*, seem to be a critical and potentially rate-limiting step during reprogramming (Mikkelson et al., 2008). ECAT genes are activated at high levels and hypomethylated at their promoters in pluripotent cells, whereas hypermethylated in somatic cells, reflecting their transcriptionally repressed state (Imamura et al., 2006). The repression of these ECAT genes and high levels of methylation of their promoters contributes to a loss of pluripotency (Imamura et al., 2006). Conversely, the formation of iPSCs involves activation of these genes and demethylation of their promoters. This difference has become widely used to monitor successful reprogramming (Mikkelson et al., 2008). Therefore, the loss of DNA methylation in pluripotency genes is a critical step to achieve complete reprogramming.

As proven by the high resolution of current techniques, the epigenetic status of iPSCs and ESCs are highly similar. It is clear that the chromatin signatures reset to an ESC-like pattern during iPSC reprogramming. Despite various trials that we discussed above to improve the efficiency of reprogramming, it is still very low, indicating the high epigenetic barriers in changing the epigenetic marks of somatic cells to pluripotent ES-like cells may be a limitation. Thus, the first approach that many research groups have tried is to lower the epigenetic barriers of reprogramming by chromatin modifications.

To lower the epigenetic barrier, many efforts have been made in using various chromatin modifiers—histone deacetylase inhibitors (Butyrate, Valproic acid, TSA), histone methyltransferase inhibitor (BIX-01294), and DNA methyltransferase inhibitor (5-azacytidine). Higher levels of histone acetylation are generally associated with

increasing gene expression and open chromatin structure. Accordingly, genome-wide gene expression profiling on early reprogramming cells treated by, for example Butyrate, confirmed upregulated expressions of genes that are highly expressed in ESCs (Liang et al., 2010). Blunt treatment of histone deacetylase inhibitors raise the global levels of histone acetylation, resulting in increasing the efficiency of the reprogramming process. Especially, treatment with Valproic acid (VPA) lowered the barrier of reprogramming by enhancing the efficiency over 100-fold and even replaced viral *cMyc* in mouse, and *CMYC* and *KLF4* in human cells (Haunfu, 2008a and 2008b), indicating that specific reprogramming factors could be eliminated, when acetylation levels were increased in cells by modulating the levels of histone acetylation. However, the identity of specific acetyltransferases involved in the modulation of the reprogramming process and the mechanism underlying the enhancement of iPSC generation are still unclear.

Another histone modifier, the small molecule named BIX-01294 thought to target the repressive histone H3K9 methyltransferase G9a, could also facilitate the reprogramming process (Shi et al., 2008a). A well-known inhibitor of DNA methyltransferase, 5-azacytidine (AZA), rescued trapped partially reprogrammed cells to iPSCs by overcomeing the roadblock of iPSC reprogramming (Mikkelson et al., 2008). Overabundant repressive modifications with these inhibitors of transcriptional repressors lead to successful iPSC generation, however, we do not know their key targets during reprogramming.

The key repressive chromatin regulator proteins in normal development are Polycomb group (PcG) proteins that form multi-protein complexes and work as

transcriptional repressors of several thousand genes governing differentiation pathways during development (Morey and Helin, 2010). ESCs lacking the function of either Polycomb-repressive complex 1 (PRC1) or PRC2 differentiate into cells of the three germ layers, whereas simultaneous loss of PRC1 and PRC2 abrogates differentiation (Leeb et al., 2010). It has not been addressed whether PcG proteins affects iPSC reprogramming, however, it will be interesting to determine whether over-expression of PcG proteins could enhance reprogramming by reducing apoptosis, because PcG proteins maintain the silencing of key senescence regulating genes, such as *Ink4/Arf*, whose depletion promotes reprogramming (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009).

In spite of these diverse treatments, the identity of the major chromatin-modifying and chromatin-binding factors involved in the iPSC conversion process is not yet known. Only recently, functional links between specific epigenetic modifiers and members of ESC core transcriptional networks in ESCs have come to light. WD repeat domain 5 (Wdr5), a core member of the mammalian Trithorax (TrxG) complex and an effector of H3K4 methylation, interacts with the pluripotency transcription factor Oct4 (Ang et al., 2011).The Oct4-Sox2-Nanog circuitry and TrxG cooperately activate transcription of key regulators of self-renewal. Furthermore, Wdr5 expression is required for efficient iPSC generation. This provides an insight to how transcriptional regulators of pluripotency may cooperate with epigenitic modulators to regulate the balance between self-renewal and differentiation.

iPSCs Application from Laboratory to Patients

Generating Disease-relevant Cell Types in vitro

For many years, work has been executed to convert pluripotent cells into target cell types. The process of guiding the differentiation of ESCs or iPSCs to target cells, named directed differentiation, is a simulation of natural development in a culture dish through successive stages of cell fate specification (Gaspard and Vanderhaeghen, 2010; Murry and Keller, 2008; Peljto and Wichterle, 2011; Schwartz et al., 2008). By stimulating extracellular signaling molecules, using the same morphogens that mediate the corresponding transitions *in vivo*, pluripotent cells can be guided to change from one cell to another until it reaches the target cells.

By identifying developmental signals, the directed differentiation protocols that turn ESCs into numerous cell types that represent all three germ layers have been developed. The addition of factors, such as bone morphogenic protein (BMP) 4, in serum free media directs mesodermal specification in ESCs (Dambrot et al., 2011; Murry and Keller, 2008). Further manipulation of this protocol generates many mesodermal cell types including hematopoietic cell types (Lengerke and Daley, 2010), cardiomyocytes (Dambrot et al., 2011), skeletal myoblasts (Barberi et al., 2007), and chondrocytes (Oldershaw et al., 2010; Toh et al., 2010).

To produce cells of the endodermal lineage, Activin A is first used to direct ESCs toward definitive endodermal fate. Insulin-producing pancreatic β-cells, the cell type lost in type I diabetes, have been derived by directed differentiation by sequentially converting the cell fates in a stepwise fashion. When Activin A is followed with retinoic

acid (RA), NOGGIN, and cyclopamine to block Sonic Hedgehog (SHH) signaling, definitive endoderms are switched to a pancreatic progenitor state (Borowiak and Melton, 2009; Kroon et al., 2008). Further effort to identify small molecules by chemical screening has been attempted to achieve a pancreatic endocrine cell fate (Chen et al., 2009). Likewise, it is possible to generate hepatocytes from definitive endoderm by the exposure of FGF 10 and RA for hepatic specification, and final maturation with fibroblast growth factor (FGF) 4, hepatocyte growth factor (HGF), and epidermal growth factor (EGF) (Basma et al., 2009; Hay et al., 2008; Touboul et al., 2010).

Ectodermal fate is considered the default differentiation path taken by pluripotent cells in the absence of other extrinsic signals that bias cells toward mesodermal and endodermal states (Levine and Brivanlou, 2007). Therefore, by blocking both Activin/Nodal/Tgf-β and BMP signaling, stem cells can be differentiated into neuroectodermal cells *in vitro* (Chambers et al., 2009). Motor neurons, whose loss in a variety of genetic and sporadic diseases or injury leads to paralysis, are one of the first neural types that were produced by directed differentiation; RA guides neural progenitors to a spinal character (Wichterle et al., 2002), then SHH induces them to a ventral neural cell fate (Jessell, 2000). The resulting differentiated cells eventually contribute a significant portion of functional motor neurons because motor neurons originate from one of the most ventral regions of the developing neural tube (Boulting et al., 2011; Lee et al., 2007). Dopaminergic neurons, which are lost in Parkinson's disease, can also be derived from pluripotent cells using SHH and FGF8, which induce neural progenitor cells (NPCs) with a mid-hindbrain character (Perrier et al., 2004).

To apply the iPSC technology in the field of regenerative medicine, many groups have generated patient-specific, disease-relevant cell types, although there are significant issues such as low efficiency for differentiation of desired cell type and safety concerns for patient application (Boulting et al., 2011; Brennand et al., 2011; Dimos et al., 2008; Ebert et al., 2009; Ku et al., 2010; Lee et al., 2009; Liu et al., 2010; Marchetto et al., 2010; Maehr et al., 2009; Park et al., 2008; Rashid et al., 2010; Seibler et al., 2011; Soldner et al., 2009; Zhang et al., 2010). The progress of iPSC reprogramming as discussed previously, coupled with further optimization of *in vitro* differentiation protocols may be necessary to derive cell types that are clinically relevant for future transplantation.

Medical Applications of iPSCs to Patients

The identification and generation of a disease-relevant cellular pathology offers unprecedented opportunities for modeling human disease *in vitro*, as well as platforms for discovering drugs in the most relevant cellular context. In addition, reprogramming technology and iPSCs have the potential to be used to model and treat human disease.

Because animal models that have been generated to study human genetic diseases often do not recapitulate the whole spectrum of disease symptoms or have difficulty in finding therapies for patients, *in vitro* disease modeling using iPSCs derived from disease-specific patient cells has been encouraging. This approach can benefit for idiopathic diseases with no known genetic cause, or that has been difficult to study due to the inaccessibility of the defected cells from live patients for many neuronal disorders.

Using iPSC technology, research groups have investigated patients' genetic background as well as disease progress from their early stages by differentiating into disease-relevant target cells from patient iPSCs, thereby elucidating the common mechanisms leading to illness. Furthermore, potential drugs that could correct a disease can be screened on the disease affected cell type, aiding in the discovery of novel therapeutic compounds.

Many iPSC lines have already been derived from patients from various diseases, including spinal muscular atrophy (SMA), familia dysautonomia, rett syndrome, fragile X syndrome, Parkinson's disease, amyotrophic lateral sclerosis (ALS), liver disorder, etc (Boulting et al., 2011; Ebert et al., 2009; Lee et al., 2009; Marchetoo et al., 2010; Park et al., 2008; Rashid et al., 2010; Soldner et al., 2009; Urbach et al., 2010).

One of the first iPSC lines that has been used for *in vitro* disease modeling is from SMA patients' fibroblasts (Ebert et al., 2009). SMA is an autosomal recessive childhood neuromuscular disease that is characterized by the loss of lower motor neurons, and is caused by a decrease in levels of survival of motor neuron (SMN) protein due to mutation in the SMN1 gene with unknown mechanism (Burghes and Beattie, 2009; Ebert and Svendsen, 2010). iPSC lines from a patient with SMA indeed showed the reduced numbers of motor neurons at later time points, demonstrating the process of iPSC reprogramming and directed differentiation recapitulated disease phenotypes.

Two compounds, tobramycine and VPA are known to increase the number of full-length SMN transcripts from the *SMN2* locus in patient-derived iPSCs (Brichta et al., 2003; Sumner et al., 2003). However, it remains to be addressed whether these

compounds could elevate SMN levels in motor neurons, thereby rescuing the motor neuron loss. In the past, researchers have screened to discover chemical compounds that may elevate SMN levels using various engineered cell lines and patient fibroblasts that are easily accessible. However, these compounds have failed in the clinic, possibly because the mechanisms that control the levels of SMN protein in human fibroblasts are substantially different from those in the target disease cell type, human motor neurons, *in vivo*. Therefore, further chemical screening may aid to identify disease-specific drugs that could have the same effect in motor neurons and thus rescue motor neuron death in patients.

Recently, patient iPSC-derived neurons from familial Parkinson's disease, which is caused by the progressive loss of midbrain dopaminergic neurons have been used to model the disease *in vitro* (Seibler et al., 2011; Nguyen et al., 2011). Familial Parkinson's disease patients carry mutations in the gene encoding PTEN-induced putative kinase 1, an outer mitochondrial membrane protein, or leucine-rich repeat kinase 2, which is the most common cause of familial Parkinson's disease. In such a case in which the disease-causing mutation is known, gene targeting could be used to repair disease-causing mutation in the DNA sequence. For precise gene targeting, new techniques have been developed to make the process more efficient by employing zinc finger nucleases that decrease the risk of off-target genetic mutation, resulting in safely repaired iPSCs (Zou et al., 2009). The gene-corrected patient-specific iPSCs could generate the healthy midbrain dopaminergic neurons through *in vitro* directed differentiation process and be transplanted into the patient's brain. Therefore, define-

factor reprogramming to pluripotency could have a broad impact from the laboratory to the clinic. Compared to SCNT or ESC fusion, it is the most practical approach by far for the generation of patient-specific iPSCs that can provide an unlimited cellular resource for regenerative medicine.

Summary

The years since Takahashi and Yamanaka's groundbreaking discovery have seen enormous advance for the safety and efficacy of iPSC reprogramming technology. The first iPSCs were generated by co-transduction with viruses that express 24 reprogramming factors. Subsequent experiments narrowed the required factors down to four. Further efforts by starting with different somatic cells, for example, NSCs other than fibroblasts enable the reduction of factors down to one, *Oct4*. For clinical application in the future, the dangerous retrovirus methods needed to be modified. Although various approaches including plasmid, protein, small molecule, etc. had been made, completely transgene-free or high efficiency was a challenge. Recently, the goal toward transgene-free iPSC generation was eventually achieved by using modified RNA encoding the reprogramming factors. However, the RNA technology still has some disadvantages—costly, laborious, and technically challenging.

Reversing the normal developmental state carries formidable epigenetic barriers. However, iPSCs, which closely resemble ESCs in epigenetic signatures could overcome these barriers to gain pluripotency. Despite rapid progress in lowering epigenetic barriers by chromatin modifications during iPSC reprogramming, many mechanistic understandings of the epigenetic processes leading to pluripotency still remain questions.

Thus, it will now be interesting to explore new approaches to develop and understand iPSC reprogramming by asking the following questions. For example, which somatic cells are the best sources for iPSCs for clinical application? Is there any other system, such as modulating developmental signaling, which can replace factors and

promote the efficiency? If DNA methylation is a final critical step to convert the somatic cells to iPSCs, does reducing DNA methylation significantly change the reprogramming dynamic? Our future efforts to answer these questions may provide better platforms for studying the mechanism, and move the field one step closer to clinical application.

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Chapter 2

Notch Inhibition Renders Both Oncogenic Transgenes and p53 Inhibition Dispensable During iPSC Reprogramming

Addendum

This chapter is adapted from the original article:
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Abstract

The reprogramming of somatic cells to pluripotency using defined transcription factors holds great promise for biomedicine. However, reprogramming remains inefficient and relies either on the use of the potentially dangerous oncogenes *KLF4* and *CMYC* or the genetic inhibition of the tumor suppressor gene *p53* (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Nakagawa et al., 2008; Okita et al., 2011; Utikal et al., 2009). We hypothesized that modulation of signal transduction pathways that control proliferation and differentiation of the target somatic cell during development might increase their reprogramming potential and provide an alternative to the genetic manipulation of pathways that control cellular transformation. Here, we show that inhibition of the Notch pathway significantly improves the efficiency of reprogramming both mouse and human keratinocytes to pluripotency. Pharmacological inhibition of Notch enabled us to routinely produce mouse and human iPSCs without the use of the oncogenes *KLF4* and *CMYC* while leaving p53 activity intact. Our findings demonstrate that controlling signal transduction cascades in the target somatic cell population can enhance their potential for reprogramming.

Introduction

Use of the potent oncogenes *KLF4* and *CMYC* in the generation of induced pluripotent stem cells (iPSCs) limits their translational utility. Currently, elimination of these genes during reprogramming requires suppression of p53 activity (Kawamura et al., 2009; Okita et al., 2011), which permits the accumulation of genetic mutations in the resulting iPSCs (Marión et al., 2009). Therefore, there remains a real need for reprogramming approaches that enable iPSC generation without the use of *KLF4* and *CMYC* while leaving p53 activity intact.

Chemical screens have identified small molecules that enhance reprogramming by modulating cell signaling in partially reprogrammed intermediates (Ichida et al., 2009) or by reinforcing the pluripotency network (Silva et al., 2008; Zhu et al., 2010). However, it is unclear if the signal transduction cascades in target somatic cell populations can be modulated in such a way as to enhance their potential for reprogramming. If so, this might provide new strategies for expanding the translational utility of lineage conversion both *in vitro* and *in vivo*.

The Notch signaling pathway is highly conserved and regulates the proliferation and differentiation of various progenitor cell types in many multicellular organisms (Artavanis-Tsakonas et al., 2010). Although it is recognized that the extent of cellular differentiation and proliferative capacity of a given somatic cell are important determinants of the efficiency by which it can give rise to iPSCs (Eminli et al., 2009; Hanna et al., 2009; Utikal et al., 2009), it has not been investigated whether forcing a population of somatic cells into a more potent “stem cell” state can increase its

reprogramming potential. Furthermore, the effects of modulating Notch signaling on reprogramming have yet to be investigated in any context.

As Notch signaling has been shown to induce the differentiation and reduce the proliferation of keratinocytes *in vivo* (Lefort et al., 2004; Rangarajan et al., 2001), we hypothesized that inhibition of Notch during keratinocyte reprogramming might promote the conversion of these somatic cells into iPS cells. We felt that keratinocytes were an attractive model system for such reprogramming studies as, if Notch inhibition did have a substantial effect on the reprogramming of this readily accessible cell type, it could be immediately translated to the production of patient-specific pluripotent stem cells (Aasen et al., 2008; Aasen et al., 2010).

Results

DAPT Promotes Mouse Keratinocyte Reprogramming and Enable to Generate iPSCs without Klf4 and cMyc.

Notch signaling is activated by the γ-secretase complex, which cleaves the membrane-bound Notch receptor upon ligand binding and generates a free intracellular domain that translocates to the nucleus and modulates transcription (Artavanis-Tsakonas et al., 2010). It has previously been shown that the γ-secretase inhibitor DAPT can block Notch signaling in mouse keratinocytes (Blanpain et al., 2006). Consistent with previous findings, when we transduced neonatal mouse keratinocytes with the iPSC reprogramming factors and treated them with 10 μM DAPT, we observed a significant increase in the abundance of the full-length Notch receptor, a reduction of the cleaved

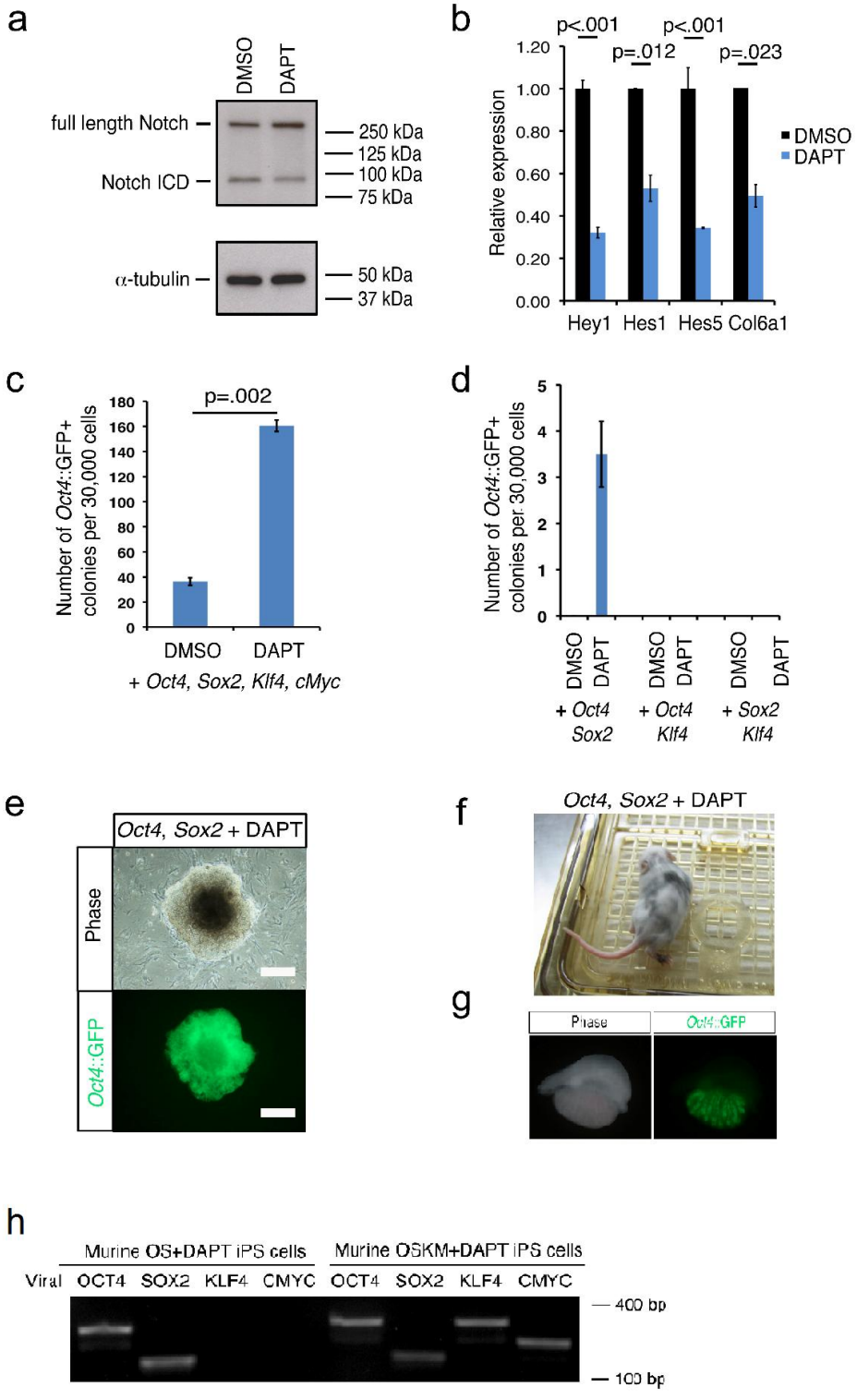
Notch intracellular domain (NICD) (Figure 2.1a), and decreased transcription of Notch-dependent target genes *Hey1*, *Hes1*, *Hes5*, and *Col6a1* (Figure 2.1b).

To determine whether inhibition of Notch could increase the efficiency of reprogramming, we transduced *Oct4::*GFP mouse keratinocytes with *Oct4*, *Sox2*, *Klf4*, and *cMyc* and cultured the resulting cells for 25 days either in the presence or absence of DAPT. We found that the addition of 10 μM DAPT led to a significant, 4-fold increase in the number of resulting GFP+ iPSC colonies (Figure 2.1c).

Because a previous study had shown that similar increase in reprogramming efficiency provided by suppression of p53 activity allowed the generation of iPSCs from keratinocytes without *Klf4* and *cMyc* (Kawamura et al., 2009), we wondered whether DAPT treatment might also replace these factors. Indeed, although transduction of *Oct4* and *Sox2* were not sufficient to induce mouse keratinocyte reprogramming, when they were combined with DAPT treatment, iPSC colonies were routinely obtained (Figures 2.1d-e). This effect was specific to *Oct4* and *Sox2*-transduced cells as other 2-factor combinations of factors did not yield iPSCs (Figure 2.1d). The mouse iPSCs generated without *Klf4* and *cMyc* harbored only the *Oct4* and *Sox2* transgenes (Figure 2.1h) and when injected into blastocysts contributed to the development of chimeric mice (Figure 2.1f), including to their germ-line (Figure 2.1g), confirming their pluripotency.

Figure 2.1. DAPT treatment promotes mouse keratinocyte reprogramming.
a, Western blot for Notch1 on *OCT4*, *SOX2*, *KLF4*, and *CMYC*-transduced mouse keratinocytes with or without 10 μM DAPT treatment for 3 days. An increase in full-length Notch and a decrease in Notch intracellular domain (Notch ICD) levels is apparent. Lines representing the molecular weight markers are shown on the right. **b,** QPCR analysis for the expression of Notch-dependent genes in mouse keratinocytes transduced with the reprogramming factors +/- 10 μM DAPT treatment for 3 days. **c,** The efficiency of *Oct4*::GFP+ iPSC generation from mouse keratinocytes transduced with *Oct4*, *Sox2*, *Klf4*, and *cMyc* with or without 10 μM DAPT treatment from days 1-18 of reprogramming. **d,** The efficiency of *Oct4*::GFP+ iPSC generation from mouse keratinocytes transduced with all combinations of 2 reprogramming factors with or without 2.5 μM DAPT treatment from days 1-18 post-transduction. **e,** A P0 mouse iPSC colony generated using *OCT4*, *SOX2*, and DAPT, scale bars = 100 μm. **f,** A chimeric mouse generated from C57BL6 *OCT4*, *SOX2* + DAPT miPSCs injected into ICR (albino) blastocysts. Black coat color is derived from the miPSCs. **g,** The E13.5 genital ridge of an embryo derived from non-transgenic blastocysts injected with *OCT4*, *SOX2* + DAPT miPSCs derived from *Oct4*::GFP keratinocytes. *Oct4*::GFP+ cells are found in the genital ridge, indicating that the miPSCs contribute to the germ line. **h,** iPSCs generated with *Oct4*, *Sox2* and DAPT do not contain *Klf4* or *cMyc* transgenes. Genomic DNA was isolated from mouse keratinocyte-derived iPSC lines and probed for transgenic insertions by PCR using primers specific for the viral reprogramming genes. Lines representing the molecular weight markers are shown on the right. For all experiments, error bars represent the standard deviation between two biological replicates and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

Figure 2.1 (Continued).

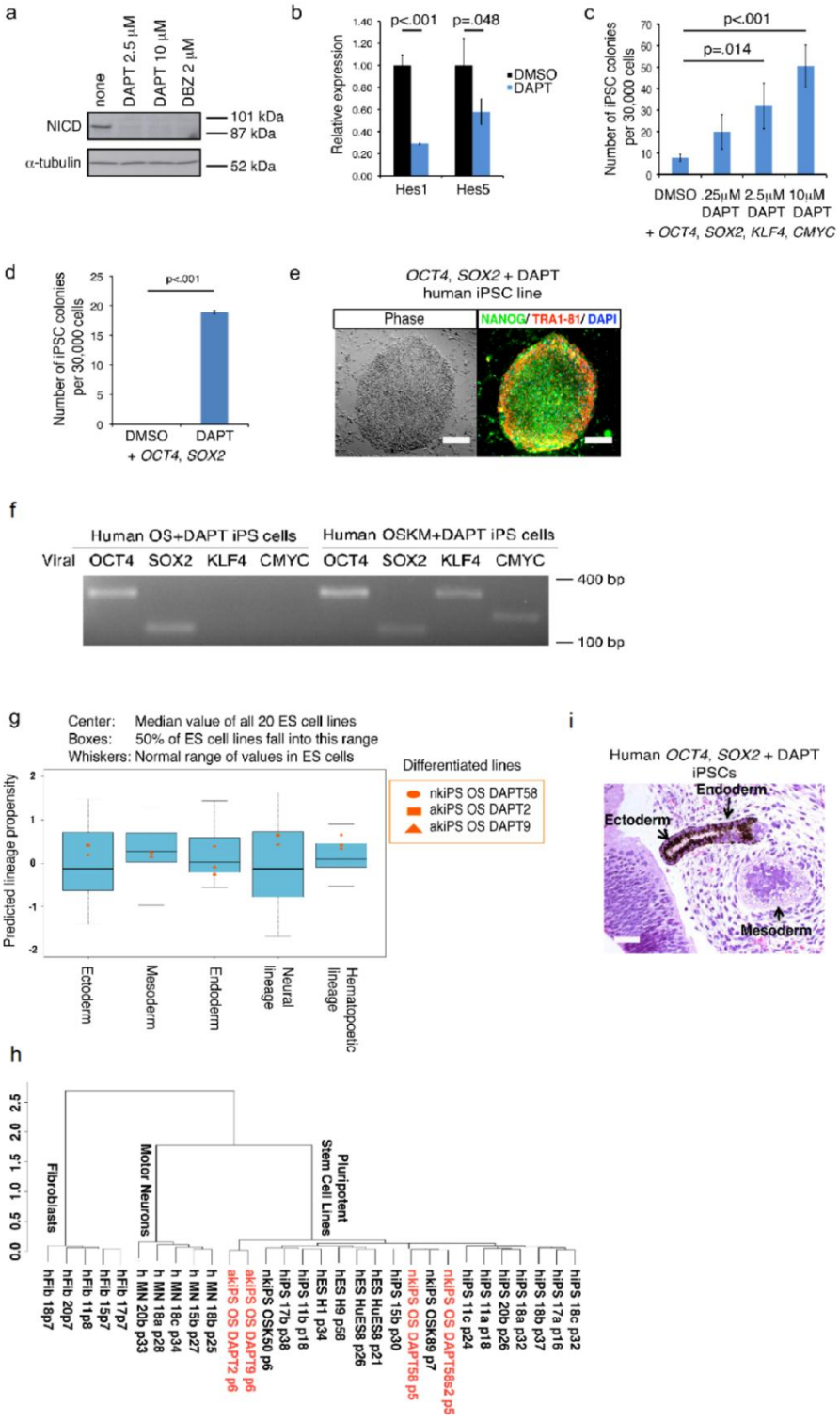


DAPT Improves the Reprogramming of Human Neonatal and Adult Keratinocytes into iPSCs and Replace KLF4 and cMYC.

Since the role of NOTCH in antagonizing the proliferation and self-renewal of keratinocytes is conserved from mouse to human (Nguyen et al., 2006), we next asked whether chemical inhibition of NOTCH could also promote the reprogramming of human keratinocytes into iPSCs. DAPT treatment of human neonatal keratinocytes that had been transduced with the iPSC reprogramming factors greatly reduced the amount of NOTCH intracellular domain (Figure 2.2a) and the transcription of the NOTCH target genes *HES1* and *HES5* (Figure 2.2b). When we transduced the human keratinocytes with reprogramming viruses and then administered DAPT, we observed a dose-dependent increase in the number of presumptive iPS cell colonies that expressed NANOG and Tra1-81 (Figure 2.2c). Similarly to the mouse keratinocytes, when we attempted to reprogram human keratinocytes with only *OCT4* and *SOX2* alone, iPSC colonies did not form (Figure 2.2d). However, when *OCT4* and *SOX2* were combined with DAPT treatment, presumptive human iPSC colonies expressing both NANOG and TRA-1-81 were readily observed (Figure 2.2d). When these colonies were expanded in culture, the resulting human cell lines continued to express both NANOG and TRA-1-81 (Figure 2.2e) suggesting that they were stable iPSCs that had been generated without *KLF4* and *CMYC* (Figure 2.2f). To determine whether these cell lines were pluripotent, we subjected them to a "scorecard" assay for pluripotency that we recently developed (Bock et al., 2011). We found that these presumptive human iPSC lines were indeed composed of pluripotent cells and that they performed comparably to human embryonic

Figure 2.2. DAPT treatment promotes human keratinocyte reprogramming.
a, Western blot detecting Notch intracellular domain (Notch ICD) expression levels in *OCT4*, *SOX2*, *KLF4*, and *CMYC*-transduced human keratinocytes +/- DAPT or DBZ treatment for 3 days. Lines representing the molecular weight markers are shown on the right. **b,** QPCR analysis for mRNA levels of NOTCH-dependent genes in human keratinocytes transduced with the reprogramming factors with or without 10 μM DAPT treatment for 3 days. **c,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation from human neonatal keratinocytes transduced with *OCT4*, *KLF4*, *SOX2*, and *CMYC* and treated with different concentrations of DAPT from days 1-18 post-transduction. **d,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation from human neonatal keratinocytes transduced with *OCT4* and *SOX2* and cultured in the presence or absence of 2.5 μM DAPT from days 1-18 post-transduction. **e,** NANOG+/TRA-1-81+ *OCT4*, *SOX2* + DAPT iPSC line generated from human neonatal keratinocytes, scale bars = 100 μm. **f,** iPSCs generated with *OCT4*, *SOX2* and DAPT do not contain *KLF4* or *CMYC* transgenes. Genomic DNA was isolated from human keratinocyte-derived iPSC lines and probed for transgenic insertions by PCR using primers specific for the viral reprogramming genes. Lines representing the molecular weight markers are shown on the right. **g,** Lineage scorecard analysis of embryoid bodies differentiated for 16 days, performed as described (Bock et al., 2011). The analysis shows that the adult and neonatal human keratinocyte-derived iPSC lines generated with *OCT4*, *SOX2*, and DAPT differentiate into endodermal, mesodermal, and ectodermal cells with propensities similar to other human pluripotent stem cell lines. The symbols indicate how this cell line compares to a reference set of 20 ESC lines for each of the dimensions covered by the lineage scorecard. **h,** Hierarchical clustering of NanoString mRNA expression profiles of DAPT iPSC lines. Hierarchical clustering of mRNA expression profiles of pluripotent stem cell lines was performed as described (Bock et al., 2011) The *OCT4*, *SOX2*, DAPT iPSCs derived from human keratinocytes are compared with other human pluripotent stem cell lines such as ESC (hES) and fibroblast- (hiPS) and keratinocyte-derived iPSC lines induced with *OCT4*, *SOX2*, and *KLF4* (nkiPSOSK50). Primary fibroblast lines (hFib) and hESC-derived motor neurons (hMN) are also included. "nkiPS OSDAPT58" is a human iPSC line generated from neonatal keratinocytes with *OCT4*, *SOX2*, and DAPT. "nkiPS OSDAPT58s2," is a subclone from passage 2 of hkiPS OSDAPT58. "akiPS OSDAPT2" and "akiPSOSDAPT9" are two independent iPSC lines derived from adult keratinocytes using *OCT4*, *SOX2*, and DAPT. **i,** Teratomas containing differentiated cells of all three germ lineages generated by iPSC derived from human neonatal kerationcytes using *OCT4*, *SOX2*, and DAPT, scale bar = 50 μm. **j,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation from human adult keratinocytes transduced with *OCT4*, *KLF4*, *SOX2*, and *CMYC* and treated with or without 10 μM DAPT from days 1-18 post-transduction. **k,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation from human adult keratinocytes transduced with *OCT4* and *SOX2* and cultured in the presence or absence of 2.5 μM DAPT from days 1-18 post-transduction. **l,** NANOG+/TRA-1-81+ iPSC line generated from human adult keratinocytes using *OCT4*, *SOX2* + DAPT, scale bars = 100 μm. For all experiments, error bars represent the standard deviation between two or three biological replicates and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

Figure 2.2. (Continued)



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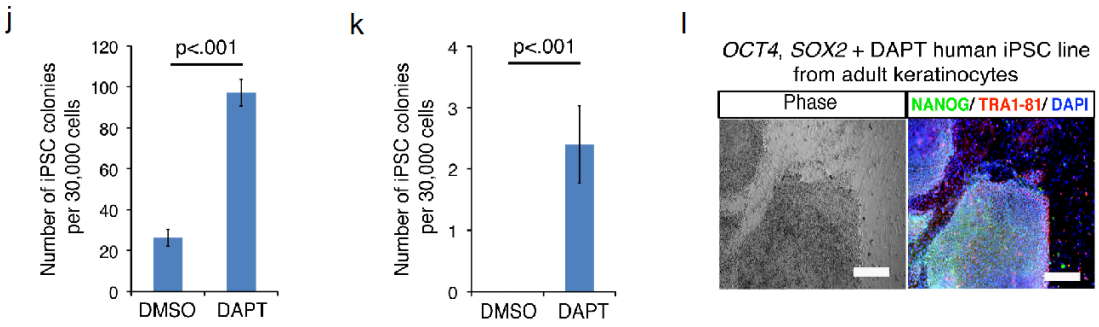


stem cells (ESCs) in their expression of pluripotency-associated genes and differentiation propensities into the three embryonic germ layers (Figures 2.2g-h). To confirm their differentiation capacity, we also injected the *OCT4*, *SOX2* + DAPT cells into immunocompromised mice. We found that they readily formed teratomas containing differentiated cells (Figure 2.2i).

Translational applications would require the DAPT-dependent generation of *KLF4* and *CMYC*-free iPSCs from adult keratinocytes. Therefore, we determined if DAPT treatment increased the reprogramming potential of adult human keratinocytes. As with mouse and human neonatal keratinocytes, we found that DAPT treatment of *KLF4*, *SOX2*, *OCT4*, and *CMYC*-transduced adult human keratinocytes significantly improved their rate of reprogramming (Figure 2.2j) and also enabled the generation of iPSCs with just *OCT4* and *SOX2* (Figures 2.2k-l). The scorecard assay again verified that these 2-factor iPSCs were pluripotent (Figures 2.2g-h). Together, these results demonstrate that DAPT reliably enables the generation of *bona fide* mouse and human iPSCs from keratinocytes without *KLF4* and *CMYC*.

DAPT Enhances Reprogramming in Keratinocytes by Specifically Inhibiting Notch Signaling.

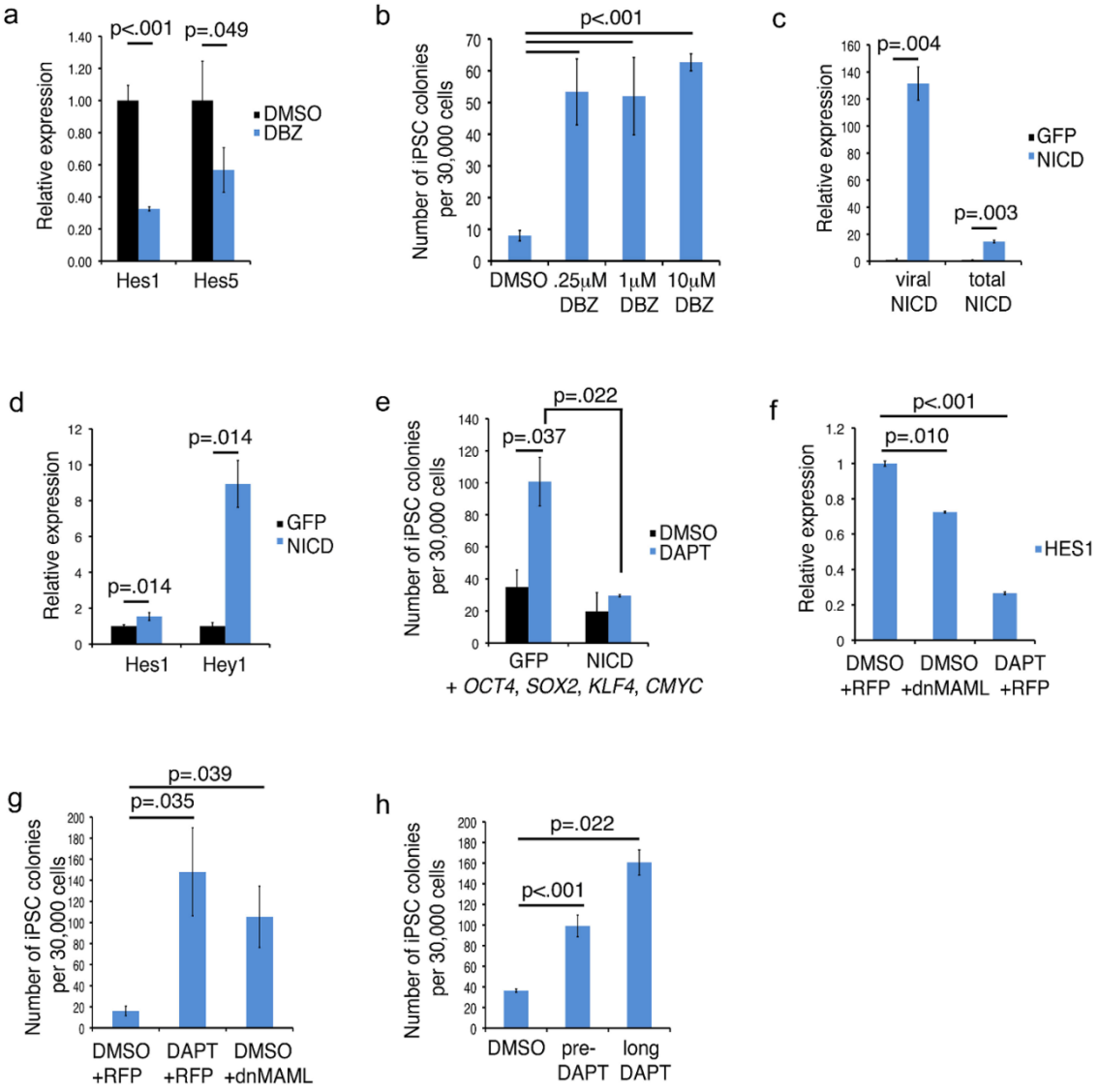
Our results thus far suggest that antagonizing Notch signaling in keratinocytes may promote their conversion into iPSCs. To begin verifying that NOTCH was indeed the functional target of DAPT during reprogramming, we first tested a structurally distinct

γ-secretase inhibitor, DBZ (Fuwa et al., 2007), for activity in iPSC generation. When we treated human keratinocytes with DBZ, we observed significant reductions in the levels of the intracellular domain of the NOTCH receptor (Figure 2.2a) and the NOTCH-dependent genes *HES1* and *HES5* (Figure 2.3a), indicating that chemical administration inhibited NOTCH signaling. Consistent with the notion that NOTCH inhibition was increasing the rate of reprogramming, DBZ significantly stimulated the formation of human iPSC colonies (Figure 2.3b).

Both DBZ and DAPT could have effects on the processing of unidentified γ-secretase substrates that are distinct from NOTCH, which might also impact reprogramming efficiency. If the beneficial effects of DAPT on reprogramming were being mediated through the specific inhibition of NOTCH signaling rather than through some other target of γ-secretase, then we reasoned that constitutive activation of NOTCH signaling should eliminate the beneficial effect of DAPT. Consistent with this notion, we found that the overexpression of the NOTCH intracellular domain (Figure 2.3c) stimulated the expression of NOTCH-target genes (Figure 2.3d) and completely eliminated the positive effects of DAPT on reprogramming (Figure 2.3e). Conversely, we reasoned that antagonizing the transcriptional activity of NOTCH should increase the rate of keratinocyte reprogramming. Indeed, when we suppressed NOTCH activity by overexpressing a dominant-negative form of *MAML1* (Figure 2.3f), a transcriptional co-activator for NOTCH (Nam et al., 2003 and 2006), we observed a significant increase in iPSC generation from keratinocytes transduced with all four reprogramming factors (Figure 2.3g). Therefore, we conclude that the inhibition of NOTCH signaling can

Figure 2.3. γ-secretase inhibition promotes reprogramming by blocking Notch signaling early in reprogramming.
a, QPCR analysis for mRNA levels of NOTCH-dependent genes in human keratinocytes transduced with the reprogramming factors with or without 2 μM DBZ treatment for 3 days. **b,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation from human neonatal keratinocytes transduced with *OCT4*, *KLF4*, *SOX2*, and *CMYC* and treated with different concentrations of DBZ from days 1-18 post-transduction. **c,** QPCR analysis for viral NOTCH intracellular domain (NICD) and total NOTCH ICD in human neonatal keratinocytes transduced with lentivirus encoding NOTCH ICD or GFP. **d,** QPCR analysis of expression levels of NOTCH-dependent genes in human neonatal keratinocytes transduced with Notch ICD or GFP. **e,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation from human neonatal keratinocytes transduced with *OCT*, *SOX2*, *KLF4*, and *CMYC* and GFP or NOTCH ICD and treated with DMSO or 10 μM DAPT from days 1-18 post-transduction. Cells were transduced with NOTCH ICD or GFP lentivirus 1 day after transduction with the reprogramming factors. **f,** QPCR analysis of expression levels of NOTCH-dependent gene HES1 in human neonatal keratinocytes transduced with dominant-negative Mastermind-like-1 (dnMAML) or RFP. **g,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation from human neonatal keratinocytes transduced with *OCT*, *SOX2*, *KLF4*, and *CMYC* and RFP or dnMAML and treated with DMSO or 10 μM DAPT from days 1-18 post-transduction. **h,** The efficiency of NANOG+/ TRA-1-81+ iPSC generation over a time course of 10 μM DAPT treatment in mouse keratinocytes transduced with *OCT4*, *SOX2*, *KLF4*, and *CMYC*. "pre-DAPT" denotes treatment from 6 days before transduction to 1 day before transduction, and "long DAPT" denotes treatment from 6 days before transduction until 18 days post-transduction. For all experiments, error bars represent the standard deviation between two-three biological replicates and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

Figure 2.3 (Continued).



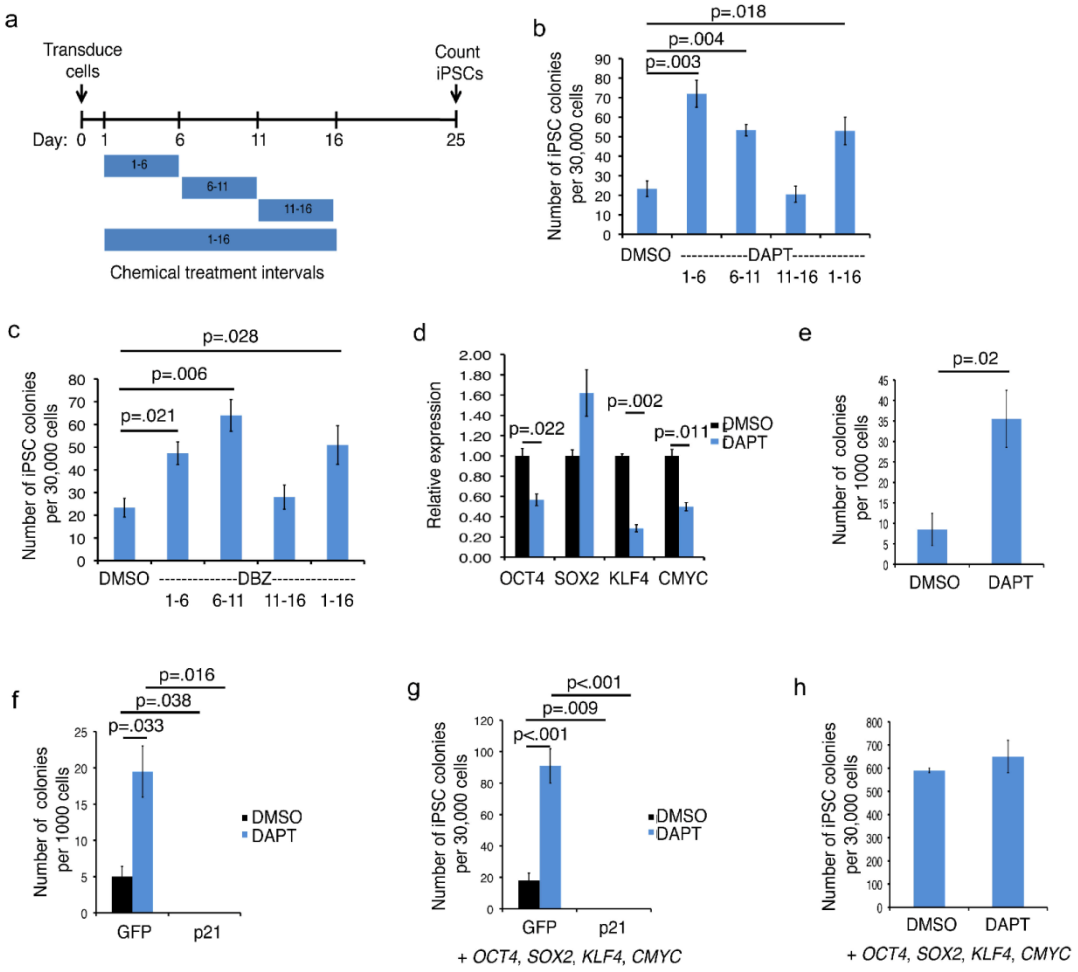
significantly increase the efficiency of reprogramming both human and mouse keratinocytes.

γ-secretase Inhibition Functions Early in Keratinocyte Reprogramming

We and others have previously identified small molecules that enhance iPSC generation by acting at late time points in the cell conversion process either on partially-reprogrammed intermediates or the iPSCs themselves (Ichida et al., 2009; Maherali et al., 2009; Silva et al., 2008; Zhu et al., 2010). In order to determine when Notch inhibition was required to promote reprogramming, we treated mouse keratinocytes with DAPT either before or both before and after transduction with all four iPSC factors. While treatment both before and after transduction yielded a 4-fold increase in iPSC generation, we found that pre-treatment alone resulted in a significant 2.5-fold enhancement in reprogramming efficiency (Figure 2.3h). These results indicate that DAPT can act on the starting keratinocytes to enhance their reprogramming potential.

To more precisely pinpoint the effective post-transduction treatment window, we transduced human keratinocytes with *KLF4*, *OCT4*, *SOX2*, and *CMYC* and administered DAPT or DBZ from days 1-6, 6-11, 11-16, or 1-16 after viral infection (Figures 2.4a-c). Chemical inhibition of NOTCH signaling was most effective during early time points, significantly increasing iPSC generation when used from days 1-6 and 6-11 (Figures 2.4b-c). In contrast, a later treatment from days 11-16 had little effect on reprogramming

Figure 2.4. Notch inhibition promotes keratinocyte reprogramming by enhancing proliferation.



a, Schematic of the post-transduction DAPT treatment time course on human neonatal keratinocytes. **b,** the efficiency of NANOG+/TRA-1-81+ iPSC generation from human neonatal keratinocytes transduced with *OCT4*, *SOX2*, *KLF4*, and *CMYC* and treated with varied intervals of 10µM DAPT or **c,** 2µM DBZ. **d,** QPCR analysis of endogenous *OCT4*, *SOX2*, *KLF4*, and *CMYC* treated with DMSO or 10µM DAPT for 3 days. **e,** Colony forming assay on human neonatal keratinocytes. After DMSO or 10µM DAPT treatment, keratinocytes were re-plated at 1000 cells/35mm dish and colonies were scored two weeks later. Colonies containing >35 cells were scored positive. **f,** Colony forming assay using human neonatal keratinocytes transduced with GFP or p21. **g,** The efficiency of NANOG+/TRA-1-81+ iPSC generation from human neonatal keratinocyts transduced with *OCT4*, *SOX2*, *KLF4*, and *CMYC* and GFP or p21 and treated with DMSO or 10µM DAPT from days 1-18 post-transduction. **h,** The efficiency of *Oct4*::GFP+ iPSC formation from MEFs transduced with *Oct4*, *Sox2*, *Klf4* and *cMyc* and treated with DMSO or 10µM DAPT from days 1-18 post-transduction. For all experiments, error bars represent the standard deviation between two-three biological replicates and statistical significance was determined using a two-tail homoscedastic Student's t-test.

(Figures 2.4b-c). Together, these results indicate that Notch inhibition can act on the starting keratinocytes and just after the initiation of transcription factor overexpression to enhance reprogramming.

Notch Inhibition does not Activate the Expression of Endogenous Reprogramming Factors

One way that Notch inhibition could promote iPSC formation is by activating the expression of the reprogramming transcription factors from their endogenous loci. However, when we treated human keratinocytes with DAPT and analyzed their gene expression, we found that levels of *KLF4*, *OCT4*, and *CMYC* actually decreased and *SOX2* did not significantly change (Figure 2.4d), indicating that Notch inhibition does not facilitate reprogramming by activating these reprogramming genes.

Notch Inhibition Promotes Keratinocyte Rreprogramming by Enhancing Proliferation.

Previous studies have shown that the replicative potential of the starting somatic cells is a key determinant of iPSC reprogramming efficiency (Hanna et al., 2009;Utikal et al., 2009). Artificially delaying the onset of senescence by inhibiting the p53-p21 signaling pathway with small RNAs promotes iPSC generation (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Okita et al., 2011; Utikal et al., 2009), possibly by creating more opportunities for reprogramming to successfully occur (Hanna et al., 2009; Utikal et al., 2009).

In the mammalian epidermis, Notch signaling regulates tissue development and homeostasis by inducing keratinocytes to exit the cell cycle and begin differentiating (Lefort et al., 2004). To determine if chemical inhibition of Notch signaling in keratinocytes might be enhancing their reprogramming potential by increasing their long-term proliferative capacity, we performed a colony-forming assay (Jones et al., 1993) on DAPT- or DMSO-treated human keratinocytes (Figure 2.4e). The ability to form large colonies on collagen demonstrates the ability of keratinocytes to self-renew extensively and is a functional property unique to undifferentiated cells of this lineage (Jones et al., 1993). In contrast, differentiated keratinocytes senesce after only a few rounds of division and do not form colonies (Jones et al., 1993). DAPT treatment of human keratinocytes for 6 days significantly increased the number of cells capable of forming large colonies when cultured for an additional 14 days in the absence of the chemical (Figure 2.4e). The resulting 4-fold increase in colony formation rate was similar in magnitude to the elevation in iPSC generation with DAPT treatment (Figure 2.2c). To determine if this increased self-renewal capacity was indeed promoting reprogramming, we transduced keratinocytes with p21 to limit replication and attempted to reprogram them either with or without DAPT. The forced p21 expression severely impaired the self-renewal potential of the keratinocytes (Figure 2.4f) and inhibited iPSC formation after transduction with the four reprogramming factors and treatment with DAPT (Figure 2.4g).

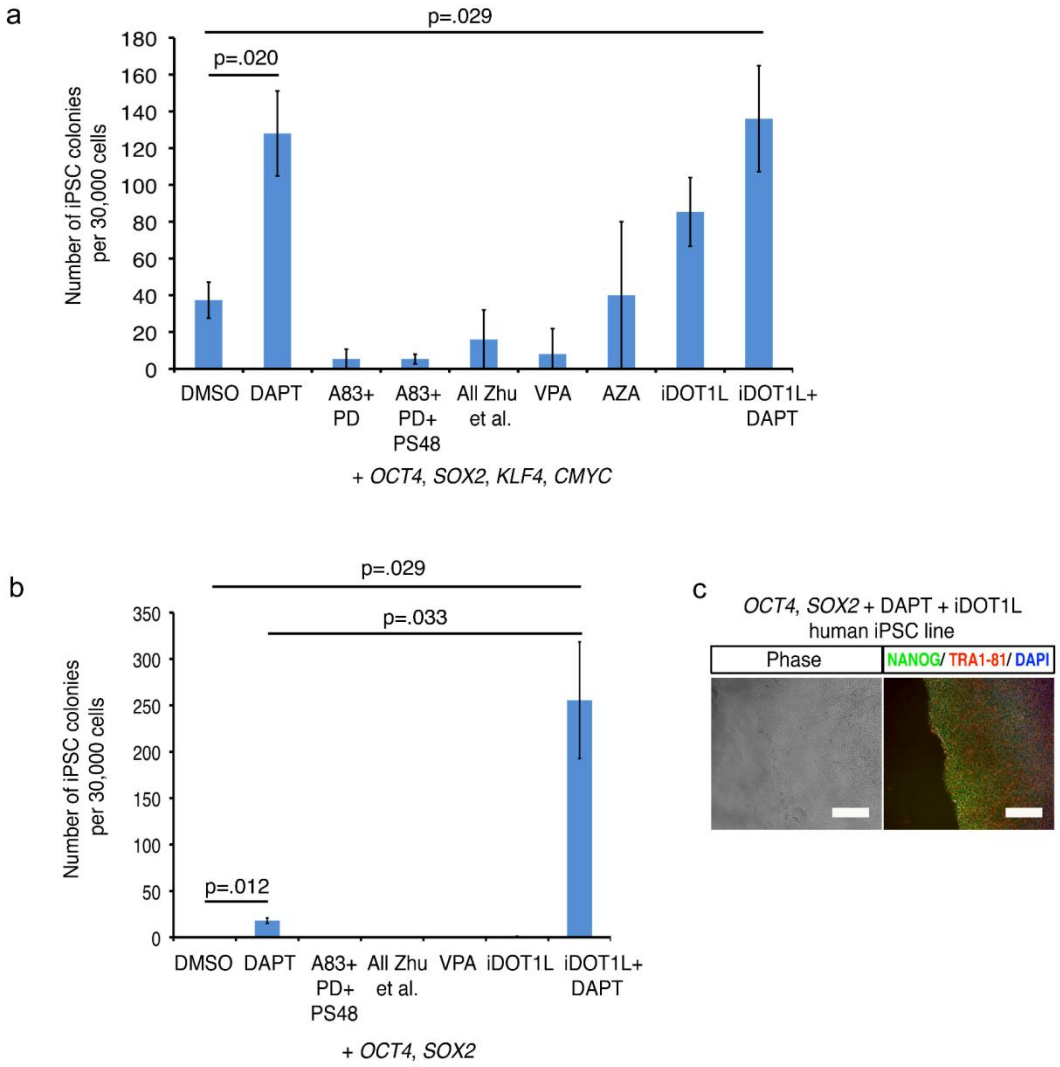
Because Notch inhibition does not promote fibroblast replication (Kavian et al., 2010), if this is the mechanism by which DAPT improves reprogramming, we would not

expect chemical treatment to affect mouse embryonic fibroblast (MEF) reprogramming. Indeed, DAPT treatment of MEFs transduced with all four reprogramming factors did not affect the rate of iPSC generation (Figure 2.4h). Together, these results suggest that Notch inhibition promotes iPSC generation from keratinocytes at least in part by enhancing their long-term replicative potential.

Synergism of DAPT and Inhibitor of DOT1L Highly Enhances Reprogramming with OCT4 and SOX2.

Knowing that Notch inhibition enhances iPSC generation through this unique mechanism, we next wanted to compare its activity to previously described reprogramming molecules that act through other mechanisms (Huangfu et al., 2008; Mikkelsen et al., 2008; Onder et al., 2012; Zhu et al., 2010) and identify any that DAPT might synergize with. When we transduced human neonatal keratinocytes with *KLF4*, *SOX2*, *OCT4*, and *CMYC* and treated them with various combinations of compounds shown to enhance reprogramming in other reports, including an activator of 3'-phosphoinositide-dependent kinase-1 (PDK1) (Zhu et al., 2010), inhibitors of TGF-β, MEK, and GSK3β signaling (Zhu et al., 2010), histone deactylase inhibitors (Huangfu et al., 2008; Zhu et al., 2010), histone methyltransferase inhibitors (Onder et al., 2012; Zhu et al., 2010), and a DNA methyltransferase inhibitor (Mikkelsen et al., 2008), we found that DAPT treatment was most potent at enhancing reprogramming (Figure 2.5a). This remained true when we attempted reprogramming with only *OCT4* and *SOX2* (Figure

Figure 2.5. Inhibition of NOTCH signaling and DOT1L activity synergize to enable high efficient reprogramming with *OCT4*and *SOX2*.



a, Comparison of NANOG+/TRA-1-81+ iPSC generation from *OCT4*, *SOX2*, *KLF4*, and *CMYC*-transduced human neonatal keratinocytes using 10µM DAPT versus other published reprogramming chemicals. "A83"= A8301 (.5µM), "PD" = PD0325901 (.5µM), "All Zhu et al."= A8301 (.5µM), PD0325901 (.5µM), PS48 (5µM), sodium butyrate (.25mM), Parnate (2µM), CHIR99021 (3µM), "AZA"=5-Aza-cytidine (.5µM), "VPA"= valproic acide (.5mM), "iDOT1L" = EPZ004777 (3µM). **b,** Comparison of NANOG+/TRA-1-81+ iPSC generation from *OCT4*-, *SOX2*-transduced human neonatal keratinocytes using 2.5µM DAPT versus other published reprogramming chemicals. **c,** iPSC line generated from human neonatal keratinocyts using *OCT4*, *SOX2*, DAPT, and iDOT1L. Scale bars=100µm. For all experiments, error bars represent the standard deviation between three biological replicates in two-three individual experiments, and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

2.5b). However, with only two factors, an inhibitor of the histone methyltransferase DOT1L (iDOT1L) synergized with DAPT to elevate the rate of iPSC generation by 10-fold over the rate with DAPT alone, making it more efficient than 4-factor reprogramming either with or without DAPT (Figure 2.5b). The *OCT4* + *SOX2* + DAPT + iDOT1L colonies could be readily expanded and maintained NANOG and TRA-181 expression (Figure 2.5c). These data indicate that Notch inhibition is a potent enhancer of reprogramming in keratinocytes that can synergize with chromatin-modifying compounds to induce pluripotency at a high efficiency with only *OCT4* and *SOX2*.

Inhibition of Notch Signaling Bypasses the Requirement of p53 Inhibition during iPSC Reprogramming

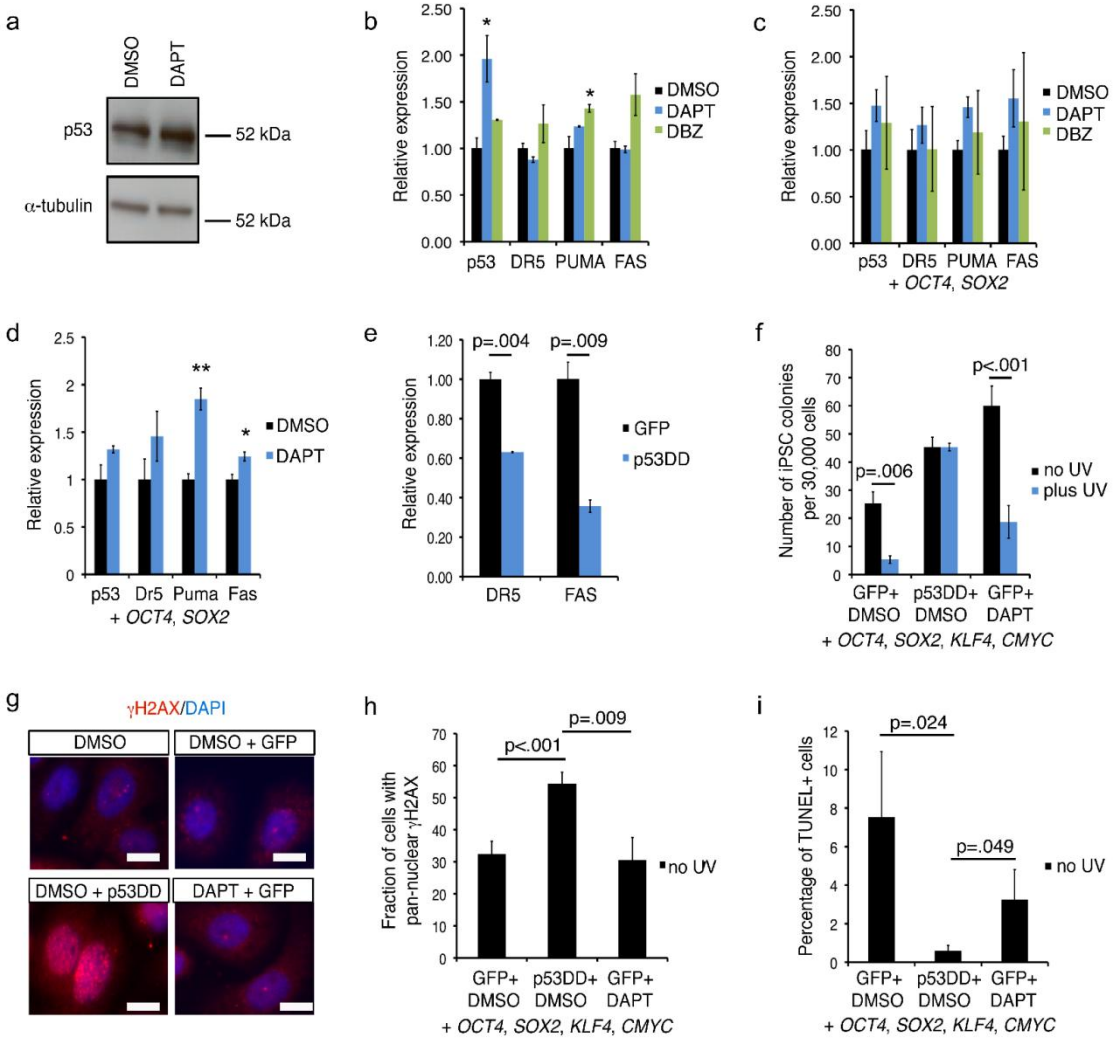
Previous studies of p53 and p21 in reprogramming have suggested that ectopic overexpression of reprogramming transcription factors can activate p53, which then induces either apoptosis or the expression of p21, thus inhibiting reprogramming (Hong et al., 2009; Kawamura et al., 2009). Because suppression of this pathway greatly facilitates iPSC generation, this approach has become an important part of reprogramming methods that reduce or eliminate integrating exogenous transcription factors (Kawamura et al., 2009; Okita et al., 2011). However, because p53 inhibition allows the accumulation of genetic mutations during reprogramming (Marión et al., 2009), alternative approaches for increasing reprogramming efficiencies would be more desirable. We therefore next asked whether Notch inhibition promotes reprogramming through a p53-dependent or independent pathway by analyzing the effects of DAPT and

DBZ treatment on p53 and several of its target genes. Interestingly, chemical inhibition of Notch signaling by DAPT or DBZ in human keratinocytes did not reduce the expression of p53 at the protein or mRNA level either before or after transduction with the reprogramming factors (Figures 2.6a-c). Moreover, transcriptional analysis of DAPT-treated human and mouse keratinocytes revealed that the mRNA levels of the p53 target genes *Dr5*, *Puma,* and *Fas* were not decreased (Figures 2.6b-d), indicating that p53 activity was not suppressed by Notch inhibition.

To confirm that DAPT treatment did not suppress p53 activity, we performed reprogramming experiments with and without DAPT after UV irradiation. UV exposure causes DNA damage, which in turn reduces reprogramming efficiencies by inducing p53-dependent apoptosis (Marión et al., 2009). p53-deficient cells, however, are resistant to the negative effects of UV irradiation on reprogramming (Marión et al., 2009). Therefore, if p53 activity was maintained in DAPT-treated cultures, then we would expect a sharp decrease in reprogramming efficiency after UV irradiation. As a control for p53-deficiency, we performed 4-factor reprogramming with or without UV irradiation using keratinocytes in which we overexpressed a dominant-negative form of p53 (p53DD) that suppressed p53 activity as evidenced by a decrease in the expression levels of p53-dependent target genes (Figure 2.6e). As expected, there was no difference in the rate of iPSC generation with or without UV exposure in this condition, indicating that p53 activity was indeed inactivated (Figure 2.6f). In contrast, without p53DD overexpression, UV exposure sharply reduced the number of iPSCs generated in DMSO-treated cultures (Figure 2.6f). Similarly, UV irradiation severely diminished the

Figure 2.6. NOTCH inhibition does not suppress p53 activity.
a, Western blot of p53 levels in human neonatal keratinocytes with DMSO or 10 μM DAPT treatment for 3 days. **b,** QPCR analysis of p53-dependent genes after 10 μM DAPT or 2 μM DBZ treatment for 3 days in untransduced human neonatal keratinocytes. **c,** QPCR analysis of p53-dependent genes after 10 μM DAPT or 2 μM DBZ treatment for 3 days in *OCT4*, *SOX2*-transduced human keratinocytes. **d,** QPCR analysis of p53-dependent genes after 10 μM DAPT treatment for 3 days in *OCT4*, *SOX2*-transduced mouse neonatal keratinocytes. **e,** QPCR analysis of p53-dependent genes in human neonatal keratinocytes with GFP or p53DD overexpression for 3 days. **f,** The efficiency of NANOG+/TRA-1-81+ iPSC generation in human neonatal keratinocytes transduced with p53DD or GFP with or without exposure to UV irradiation. **g,** γH2AX immunostaining in human neonatal keratinocytes 10 days after transduction with *OCT4*, *SOX2*, *KLF4*, and *CMYC*. **h,** Quantification of pan-nuclear γH2AX immunostaining in human neonatal keratinocytes 10 days after transduction with *OCT4*, *SOX2*, *KLF4*, and *CMYC*. **i,** The percentage of TUNEL-positive cells in human neonatal keratinocyte reprogramming cultures with active or inactive p53 (p53DD expression) 10 days after transduction with *OCT4*, *SOX2*, *KLF4*, and *CMYC*. For all experiments, error bars represent the standard deviation between two biological replicates and statistical significance was determined using a two-tailed homoscedastic Student's t-test. * denotes significance p-value < .05. ** denotes significance p-value < .01.

Figure 2.6 (Continued).



number of iPSC colonies in DAPT-treated cultures, indicating that Notch inhibition does not suppress p53 activity during reprogramming (Figure 2.6f).

Although the difference in reprogramming efficiency in p53-deficient versus DAPT-treated keratinocytes was clearly evident when UV irradiation was used to induce DNA damage, we next determined whether DNA damage was measurably influenced by DAPT treatment under normal reprogramming conditions. To test this, we quantified phosphorylated histone H2AX (γH2AX) expression in 4-factor-transduced human keratinocytes treated with DAPT or p53DD. Histone H2AX becomes phosphorylated in response to double strand DNA breaks, making it a reliable marker of DNA damage (Marión et al., 2009). Pan-nuclear γH2AX expression results from replication-induced damage and therefore would indicate insults sustained during reprogramming (Marión et al., 2009). We found that 10 days after transduction, pan-nuclear γH2AX staining was present at significantly higher numbers in p53-deficient cultures than control cultures, which is consistent with a previous study in which elevated rates of DNA damage was observed in p53-deficient cells during reprogramming and in the resulting iPSCs (Marión *et al*. 2009) (Figures 2.6g-h). The DAPT-treated cells, however, maintained a low fraction of cells with pan-nuclear γH2AX expression that was similar to the control cultures (Figures 2.6g-h). These results suggest that, in contrast to p53-deficiency, DAPT treatment does not promote the survival and reprogramming of cells with DNA damage.

To confirm that Notch inhibition does not prevent the apoptosis of compromised cells during reprogramming, we measured the fraction of TUNEL-positive nuclei in

DAPT-treated cultures. Despite high rates of DNA damage in the p53-deficient reprogramming cultures, the percentage of TUNEL-positive nuclei was greatly reduced compared to a wild-type control, indicating that inactivation of p53 likely permitted the survival of cells with compromised genomes (Figure 2.6i). In contrast, the percentage of TUNEL-positive cells was not significantly reduced by DAPT treatment (Figure 2.6i). Together, these experiments show that DNA damage is present during normal reprogramming conditions and that inhibition of p53 allows cells with damaged genomic material to persist. In contrast, DAPT-mediated Notch inhibition enhances reprogramming without facilitating iPSC generation from cells with compromised genomic integrity or promoting the survival of iPS cells that have undergone DNA damage.

Discussion

In summary, our findings suggest that signaling through the Notch pathway is a significant impediment to the early stages of the reprogramming of both mouse and human keratinocytes into iPSCs. Importantly, the mechanism by which Notch signaling likely inhibits reprogramming in both mouse and human is by limiting long-term self-renewal through a p53-independent pathway. Consistent with this hypothesis, treatment of reprogramming cultures with the γ-secretase inhibitors DAPT and DPZ reduced the levels of intracellular Notch and increased colony forming potential, leading to an increase in the rate of iPSC formation. Importantly, the resulting improvement in

reprogramming activity and the ability to generate iPSCs without the oncogenes *CMYC* and *KLF4* did not come at the expense of a reduction in p53 activity.

Our findings therefore have immediate and practical ramifications for the improved production of patient-specific human iPSCs. When taken together, our studies show that through pharmacological inhibition of NOTCH, it is routinely possible to produce human iPS cells with only *OCT4* and *SOX2*, rendering *CMYC* and *KLF4* dispensable and thereby reducing the oncogenic potential of the resulting cells. Furthermore, our findings enabled *CMYC* and *KLF4*-free iPSC production without inhibition of p53 or its target genes involved in apoptosis, allowing pro-apoptotic pathways that ensure genomic integrity to be engaged (Marión et al., 2009). Thus, in this approach, the production of oncogene free iPS cell lines does not come at the expense of an increase in mutational load (Gore et al., 2011; Marión et al., 2009).

Our findings with Notch demonstrate that developmental signaling pathways can substantially modulate the reprogramming potential of somatic cells, affecting both the quality and quantity of the resulting cell conversions. Because this approach is based on the modulation of developmental and homeostatic signaling pathways, it may also enhance the plasticity of somatic cells *in vivo*, thereby enabling efficient reprogramming *in situ* for the regeneration of diseased or damaged tissues. These findings suggest that wider investigation of how such pathways modulate the outcome of reprogramming is warranted.

Methods Summary

Oct4::GFP neonatal mouse keratinocytes were isolated from P1-P2 pups and cultured in SFM medium (Invitrogen) on collagen IV-coated plates. Neonatal human epidermal keratinocytes (Lonza) were cultured in Epilife medium (Invitrogen) on collagen-coated plates. Keratinocytes were reprogrammed using retroviruses containing *OCT4, SOX2, KLF4,* and *CMYC* produced in the pMXs backbone. Chemical treatment was initiated 1-2 days after viral transduction and re-administered every other day until the end of the experiment unless otherwise specified. DAPT was used at 10 μM for reprogramming experiments using *OCT4*, *SOX2*, *KLF4*, and *CMYC* and 2.5 μM for *OCT4*, *SOX2* reprogramming experiments unless otherwise noted. DBZ was used at 2 μM. Irradiated mouse embryonic fibroblast feeders were added 6 days after transduction and the media was changed to mouse or human embryonic stem cell medium at that time. Illumina MouseRef-8 microarrays were used for genome-wide mRNA expression analysis. SYBR green (Bio-rad) was used for QPCR analysis. Antibodies detecting mouse Notch (Santa Cruz Biotechnology, sc-6015) and cleaved human NOTCH (Cell Signaling Technology, 2421) were used for western blot analysis. Blots were quantified using ImageJ software. Antibodies specific for NANOG (Abcam, AF1997) and TRA-1-81 (Chemicon, MAB4381) were used to identify human iPSCs. Nanostring and scorecard analysis was performed as described (Bock *et al*. 2011). *In vitro* differentiation, chimera, and teratoma assays were performed as described (Ichida *et al*. 2009). UV irradiation was performed at a dosage of 30 J. Notch antibodies (ab27526, Abcam and sc-23307, Santa Cruz) were used for western blots. A γH2AX (Abcam,

ab11175) antibody was used to detect γH2AX foci. Cells in which γH2AX staining covered greater than half the nucleus were scored as positive for γH2AX foci. TUNEL staining was performed using the TUNEL kit from Pharmacia Biosciences.

Acknowledgements

The authors would like to thank E. Son for assistance with microarray data analysis, S. Sato for assistance with chimera experiments, E. Kiskinis for assistance with nanostring analysis, and K. Koszka and M. Yamaki for assistance with teratoma experiments. The authors are grateful for the financial support that made this work possible. K.E. was supported by NIH R01 grant HD045732-03, NIH P01 grant GM099117, the Howard Hughes Medical Institute, and the MacArthur Foundation. A.M was supported by NIH P01 grant GM099117. H.A. was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, Grant-in-aid for Scientific Research (21390456) and Grant-in-Aid for challenging Exploratory Research (22659304), and a grant from JST-CREST. J.I. was supported by NIH K99/R01 grant 1K99NS077435-01A1 and the Novartis Institutes for BioMedical Research. C.B. was supported by a Feodor Lynen Fellowship from the Alexander von Humboldt Foundation.

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Chapter 3

Reduction of DNMT1 protein enhances the kinetics and efficiency of iPSC reprogramming

Addendum

This chapter is adapted from the original article:
Julia E. Chung*, Camille Sindhu, Rahul Karnik, Zack Smith, Alexander Meissner, Kevin Eggan (2013). Reduction of DNMT1 protein enhances the kinetics and efficiency of iPSC reprogramming.

Abstract

Reprogramming somatic cells into induced pluirpotent stem cells (iPSCs) by defined transcription factors holds great potential for biomedicine. However, the reprogramming process retains slow kinetics and low efficiency. Reprogramming of somatic cells into iPSCs resets the epigenome to an embryonic-like state, which includes DNA demethylation of somatically-methylated pluripotentcy genes. We hypothesized that lowering the activity of the DNA methyltransferase 1 (*Dnmt1*) gene might increase the reprogramming potential and perturb a blockade of iPSC reprogramming. Here, we have generated an allelic series of *Dnmt1* mouse embryonic fibroblasts (MEFs), which have differentially modulated *Dnmt1* expression, and investigated their reprogramming efficiency and dynamics. At the epigenetic level, methylation levels of *Oct4* regulatory regions are decreased by 50% in hypomorphic *Dnmt1*MEFs, which reduces Dnmt1 expression to 10% of wild-type levels, indicating that reducing *Dnmt1*unravels the repressive effect in the *Oct4* region. We found that a 90% reduction in *Dnmt1* levels enhanced the efficiency of reprogramming over 4-fold. Importantly, hypomorphic allele ($Dnmt1^{chip}$) transduced line, which induces relatively low expression levels (20-40%) of DNMT1are reprogrammed to pluripotency with high efficiency and fast kinetics by directly activating Oct4 expression and bypassing the intermediate states. Furthermore, DNA demethylation synergized with histone acetylation and histone demethylation increased their conversion rates over 100-fold. Our findings demonstrate that DNA demethylation, by decreasing Dnmt1 expression levels, can increase the reprogramming

potential of somatic cells. Collectively, our data offers new insights into the nature of epigenetic events inherent to cellular reprogramming.

Introduction

Mouse and human somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) by ectopic over-expression of four transcription factors (*Oct4, Sox2, Klf4* and *c-Myc*) (Takahashi and Yamanaki., 2006). iPSC technology could be an attractive resource for patient-specific stem cells, however, the slow kinetics and low efficiency of reprogramming is a hurdle for iPSC generation.

The unique patterns of DNA methylation and histone modifications contribute to the epigenetic state of a cell, which is in part responsible for cell or tissue-specific gene expression. To achieve a pluripotent state during reprogramming, the removal of repressive DNA methylation at the promoter of pluripotency genes, such as *Oct4* or *Nanog* is necessary, as well as appropriate histone modifications to activate pluripotency genes and to repress somatic genes (Lister et al., 2009; Mikkelesen et al., 2008). Thus, iPSC reprogramming must involve mechanisms responsible for changing the epigenetic status of both, DNA and histones genome-wide. Recently, it has been found that changes of DNA methylation occur predominantly at the end of reprogramming during the entire spectrum of iPSC generation, suggesting that DNA methylation changes are final critical events to convert the cell type (Polo et al., 2012). In this regard, we hypothesized DNA methylation may play a critical role in reprogramming.

There are two major epigenetic regulatory systems that control DNA methylation patterns in somatic cells; DNA methyltransferase3a (*Dnmt3a*) and *Dnmt3b* mediate *de novo* DNA methylation during development, subsequently DNA methyltransferase1 (*Dnmt1*) maintains DNA methylation patterns of somatic genome (Li et al., 1992; Okano

et al., 1999). Mouse embryonic fibroblasts (MEFs) that are deficient in the *de novo* DNA methytransferase Dnmt3a and Dnmt3b enable to reprogram to pluripotency with a similar efficiency of reprogramming with wild-type cells (Pawlak et al., 2011), suggesting that *de novo* DNA methylation is dispensable for iPSC reprogramming. However, knocking out *Dnmt1* leads to embryonic lethality (Li et al., 1992) and is not tolerated in somatic fibroblasts (Jackson-Grusby et al., 2001), indicating the importance of maintaining global DNA methylation pattern for survival of somatic cells. Interestingly, it has been reported that transient inhibition of *Dnmt1*or treatment of DNA methyltransferase inhibitor 5-aza-cytidine (AZA) facilitates reprogramming of trapped intermediates (Mikkelesen et al., 2008), indicating that DNA demethylation of one or more (unknown) loci by reduced level of *Dnmt1* is a critical step in the later stage of iPSC reprogramming, and inhibition of Dnmt1 lowers the kinetic barriers of reprogramming. Another supporting report for a hypomorphic *Dnmt1* gene contribution in reprogramming is the hypomorphic allele of *Dnmt1* donor fibroblasts increases the efficiency of embryonic stem cell (ESC) derivation by somatic cell nuclear transfer (Blelloch et al., 2006). Therefore, we need to understand how much levels of reduced Dnmt1 affects the efficient reprogramming process.

Although AZA can be utilized to confer hypomethylation of DNA at low doses, it is cytotoxic to cells due to its incorporation into DNA or RNA and apparent interactions with protein biosynthesis. Therefore, it is difficult to measure the extent to which the Dnmt1 inhibition function of compound AZA treatment affects the efficiency of iPSC reprogramming. To understand how much of DNA hypomethylation achieved

by different levels of Dnmt1 expression contributes to reprogramming, we need to establish mice carrying differentially reduced levels of *Dnmt1*. Here, we have generated an allelic series of *Dnmt1 Oct4*::GFP reporter MEFs—with 100%, 60%, 50%, 20% and 10% Dnmt1 expression levels compared to wild type MEFs—using a conditional mutation in the *Dnmt1*gene and a hypomorphic allele (Gaudet et al., 2003; Jackson-Grusby et al., 2001), and converted them into iPSCs with four reprogramming factors. Using this transgenic approach, we have investigated whether the rate of iPSC generation was influenced by reducing *Dnmt1* levels, and how the reprogramming processes was altered based on comparisons with the normal iPSC reprogramming trajectory. Although further studies of the global methylation status in the differentially modulated Dnmt1 cell lines are required, our findings give us insight into how significantly DNA demethylation, which lowers the epigenetic barriers, influences in iPSC reprogramming.

Results

Generation of an Allelic Series of Dnmt1 Oct4::GFP reporter MEFs Offers Highly Modulated Dnmt1 Expression Level—100%, 60%, 50%, 20%, and 10% of Dnmt1 Expression

A mouse carrying a conditional mutation in the *Dnmt1* gene, referred to as $Dnmt1^{1lox}$, has been reported which results in the deletion of the motif for localization and the entire catalytic domain of the protein upon Cre mediated recombination (Jackson-Grusby et al., 2001). A mouse carrying a hypomorphic *Dnmt1* allele, $Dnmt1^{chip}$,

reduces Dnmt1 expression to 10% of wild-type levels and results in substantial genome-wide hypomethylation in all tissue (Gaudet et al., 2003). Using this conditional mutation and a hypomorphic allele, we were able to generate mice carrying *Dnmt1*$^{chip/1lox}$; *Oct4*::GFP reporter compound heterozygotes with substantially reduced levels of Dnmt1. First, we crossbreed *Dnmt1*$^{2lox/2lox}$ with mice carrying homologous Cre alleles to generate *Dnmt1*$^{+/1lox}$. Next we mated these mice with *Oct4*::GFP homozygotes to create reporter mice, then crossed *Dnmt1*$^{+/1lox}$; *Oct4*::GFP with hypomorphic alleles *Dnmt1*$^{chip/chip}$ to generate *Dnmt1*$^{chip/1lox}$; *Oct4*::GFP (Fig 3.1a). In addition, to generate 60% (*Dnmt1*$^{+/chip}$) and 20% (*Dnmt1*$^{chip/chip}$) Dnmt1 expressing MEFs, we crossed *Dnmt1*$^{+/chip}$; *Oct4*::GFP mice (Figure 3.1a).

From these crossbreeding strategies, we created mice containing various combinations of the hypomorphic *Dnmt1* allele and conditional mutant allele, including wild-type control: 100%, 60%, 50%, 20%, and 10% of Dnmt1 expression levels (Figure 3.1a). We harvested wild-type *Dnmt1*, *Dnmt1*$^{+/chip}$, *Dnmt1*$^{+/1lox}$, *Dnmt1*$^{chip/chip}$ and *Dnmt1*$^{chip/1lox}$; *Oct4*::GFP reporter MEFs from 13.5 day embryos. To confirm whether the genotyping results of the allelic series provides targets that express expected *Dnmt1* gene expression levels, we performed RT-qPCR for *Dnmt1*. Introduction of the *Dnmt1* mutation gradually decreased the levels of the *Dnmt1* gene compared to wild-type control and mouse ESC control (Figure 3.1b-c). As expected, *Dnmt1*$^{chip/1lox}$ MEFs have a 90% reduction in *Dnmt1* gene expression (Figure 3.1b-c). QPCR results for *Dnmt1* expression in exon 4 (Figure 3.1b) and exon 12-14 regions (Figure 3.1c) were highly similar, indicating that mutations of the *Dnmt1* gene in MEFs are consistently well-

introduced. Next, to determine whether sequentially modulated *Dnmt1* expression directly affects the amount of Dnmt1 protein expression, we performed Western blot analysis for Dnmt1 with protein extracts from each allelic series of MEFs. Consistent with the previous report (Gaudet et al., 2003; Jackson-Grusby et al., 2001), *Dnmt1*$^{chip/1lox}$ MEFs have a highly reduced level of the DNMT1 protein, whereas *Dnmt1*$^{+/1lox}$ MEFs express the DNMT1 protein at levels similar to wild-type control (Figures 3.1d-e and Table 3.1). Interestingly, all cell lines harboring the hypomorphic *Dnmt1*^{chip} allele, for example *Dnmt1*$^{+/chip}$ and *Dnmt1*$^{chip/1lox}$ MEF lines, have highly reduced protein levels of DNMT1as compared to wild-type control or *Dnmt1*$^{+/1lox}$ MEFs (Figures 3.1d-e and Table 3.1). Although the gene expression level of *Dnmt1*$^{+/chip}$ is 10% higher than that of *Dnmt1*$^{+/1lox}$ MEFs as we modulated, the protein expression level of *Dnmt1*$^{+/chip}$ MEFs were reduced more than 50% compared to *Dnmt1*$^{+/1lox}$ MEFs (Figures 3.1d-e and Table 3.1), indicating that *Dnmt1*$^{+/chip}$ and *Dnmt1*$^{+/1lox}$ MEFs might behave quite differently during the reprogramming process.

Reduced Levels of Dnmt1Expression Unravel the Repressive Effect in Endogenous Oct4 Regulatory Regions

During differentiation, the promoters of pluripotency genes become highly methylated through *de novo* methylation, ultimately reaching complete repression of the genes in somatic cells (Ben-Shushan et al., 1995; Gidekel et al., 2002). Global methylation patterns by Southern blot analysis of IAPs show extensive hypomethylation

Figure 3.1. The allelic series of *Dnmt1*;*Oct4*::GFP MEFs were sensitively modulated in *Dnmt1* expressions.
a, The cross-breed strategy generates the allelic series of *Dnmt1Oct4*::GFP reporter MEFs. **b,** QPCR analysis for the expression of *Dnmt1* Exon 4 gene in the allelic series of *Dnmt1* MEFs. The allelic series of *Dnmt1* MEFs express gradually reduced levels of Dnmt1 mRNA expression as compared to wild-type control. **c,** QPCR for *Dnmt1* Exon 12-14 regions in the allelic series of *Dnmt1* MEFs. The overall *Dnmt1* expression levels of Exon 12-14 are highly similar to that of Exon 4. **d,** Western blot for Dnmt1 on the allelic series of *Dnmt1* MEFs. The *chip* gene transduced genotypes suppress Dnmt1 protein levels as compared to wild-type or *Dnmt1*$^{+/1ox}$ genotype. Lines representing the molecular weight markers are shown on the left. **e,** Quantification of western blots for Dnmt1 on the allelic series of *Dnmt1* MEFs. For all experiments, error bars represent the standard deviation between three biological replicates in three individual experiments, and statistical significance was determined using a two-tailed homoscedastic Student's t-test (p-value < .05).

Figure 3.1. (Continued)

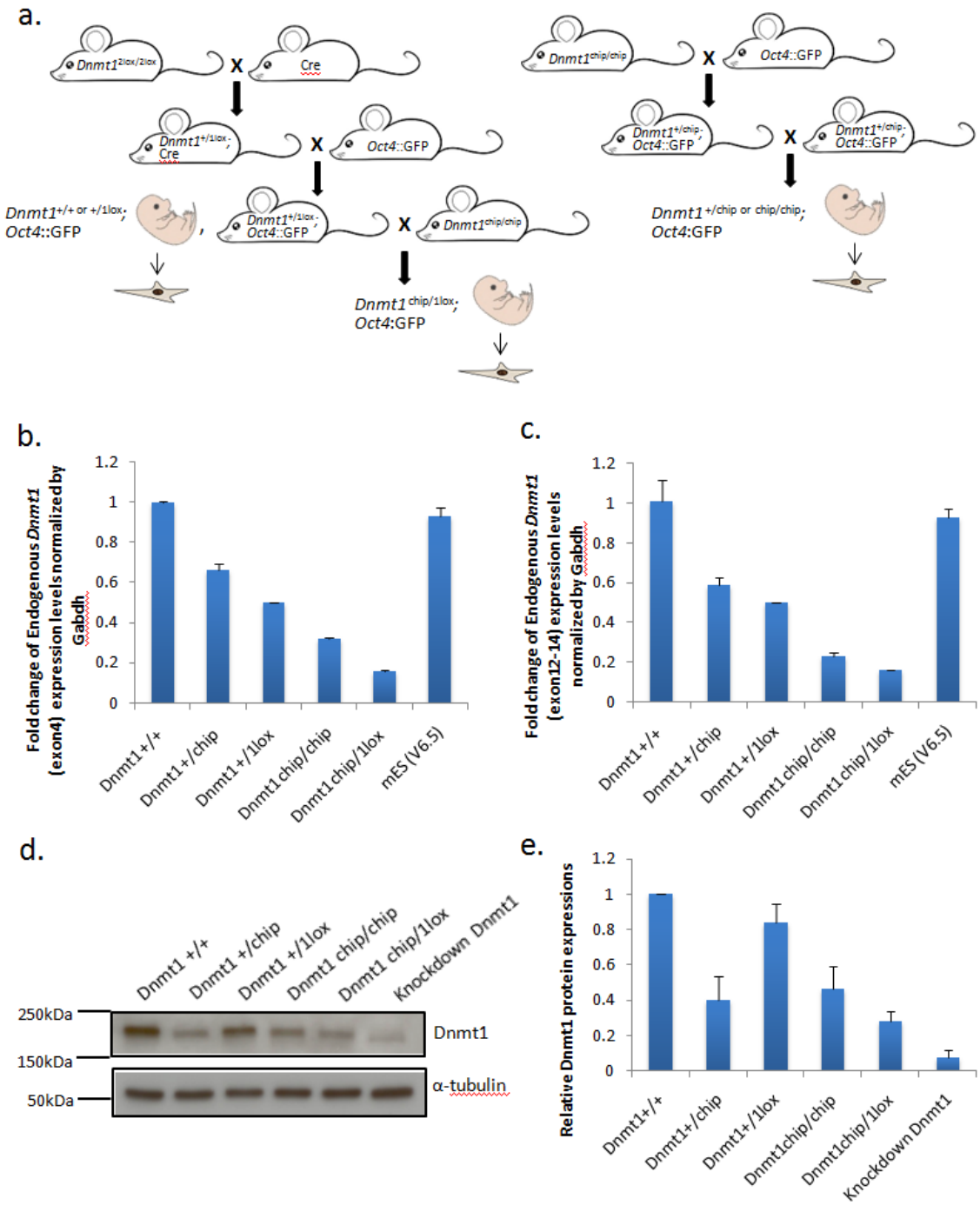


Table 3.1. Comparison of projected levels of DNMT1 protein expression versus actual levels of DNMT1 protein expression.

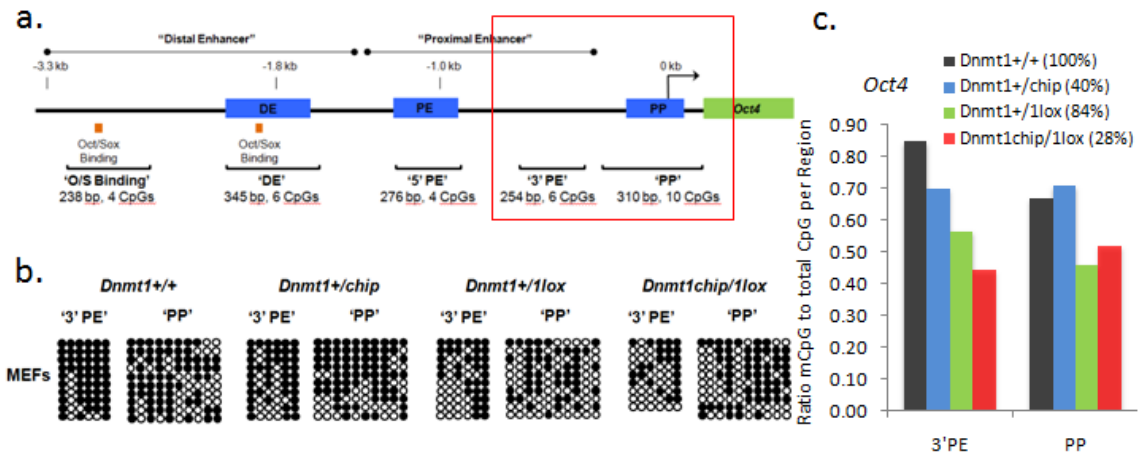
Genotype	Projected levels of DNMT1 %	Actual levels of DNMT1 %
Dnmt1$^{+/+}$	100	100 (±0)
Dnmt1$^{+/chip}$	60	40 (±13)
Dnmt1$^{+/1lox\ (+/-)}$	50	84 (±10)
Dnmt1$^{chip/chip}$	20	46 (±13)
Dnmt1$^{chip/1lox\ (chip/-)}$	10	28 (±5)

The projected levels of DNMT1 is estimated by the gene expression levels of Dnmt1, however the actual levels of DNMT1 measuared by Western blotting is not depended on the gene expressions. Any *Dnmt1*^{chip} allele introduced lines possess lower expression levels of DNMT1 than wild-type MEFs.

in both Dnmt1 deficient ESCs and hypomorphic Dnmt1 MEFs (Gaudet et al., 2003; Jackson-Grusby et al., 2001). However, there is an interesting report that shows by Southern blotting that the *Oct4* gene continues to be highly methylated in Dnmt1 knockout proliferating fibroblasts, despite the fact that many other genes become demethylated (Feldman et al., 2006). This result is presumably because of the recruitment of *de novo* methyltransferases to the *Oct4* promoter (Feldman et al., 2006). Consistent with this observation, the reactivation of Oct4 and probably its demethylation occur at a very late stage of reprogramming (Brambrink et al., 2008; Polo et al., 2012; Stadtfeld et al., 2008) Having a sense that one of the main pluripotency genes, *Oct4*, is the epigenetic barrier in iPSC reprogramming, it will be interesting to investigate how much DNA methylation in the *Oct4* promoter or enhancer region is removed depending on the amount of Dnmt1 expression in our allelic series of *Dnmt1* MEFs.

To understand the methylation dynamics at the regulatory regions of the *Oct4* locus, we examined the epigenetic state of known regulatory elements at and around the Oct4 promoter. Two regions were selected, covering six CpG dinucleotides near the 3' end of the proximal enhancer (PE) (Yeom et al., 1996) and 10 CpGs in the proximal promoter (PP), respectively (Figure 3.2a). DNA methylation at these regulatory regions was determined by bisulfate sequencing in the allelic series of *Dnmt1* MEFs. DNA methylation levels were reduced at both regions in $Dnmt1^{chip/1lox}$ MEFs, exhibiting a near 50% decrease in methylation at the 3'PE, compared to the wild-type control (Figures 3.2b-c). Overall, a reduction in DNA methylation levels at these regulatory regions

Figure 3.2. Reduced Dnmt1 levels decreased the methylation level of endogenous Oct4 regulatory regions.



a, Sequence of Oct4 promoter and enhancer regions. DNA methylation analysis is focused on major regulatory regions including Oct4 proximal promoter (PP) and 3' proximal enhancer (3' PE) regions. **b,** DNA methylation analysis for Oct4 regulatory regions. Empty circles indicate unmethylated and filled circles methylated CpG dinucleotides. c, Quantification of DNA methylation analysis. The hypomorphic *Dnmt1* MEFs highly demethylated in both 3'PE and PP region as compared to wild-type control.

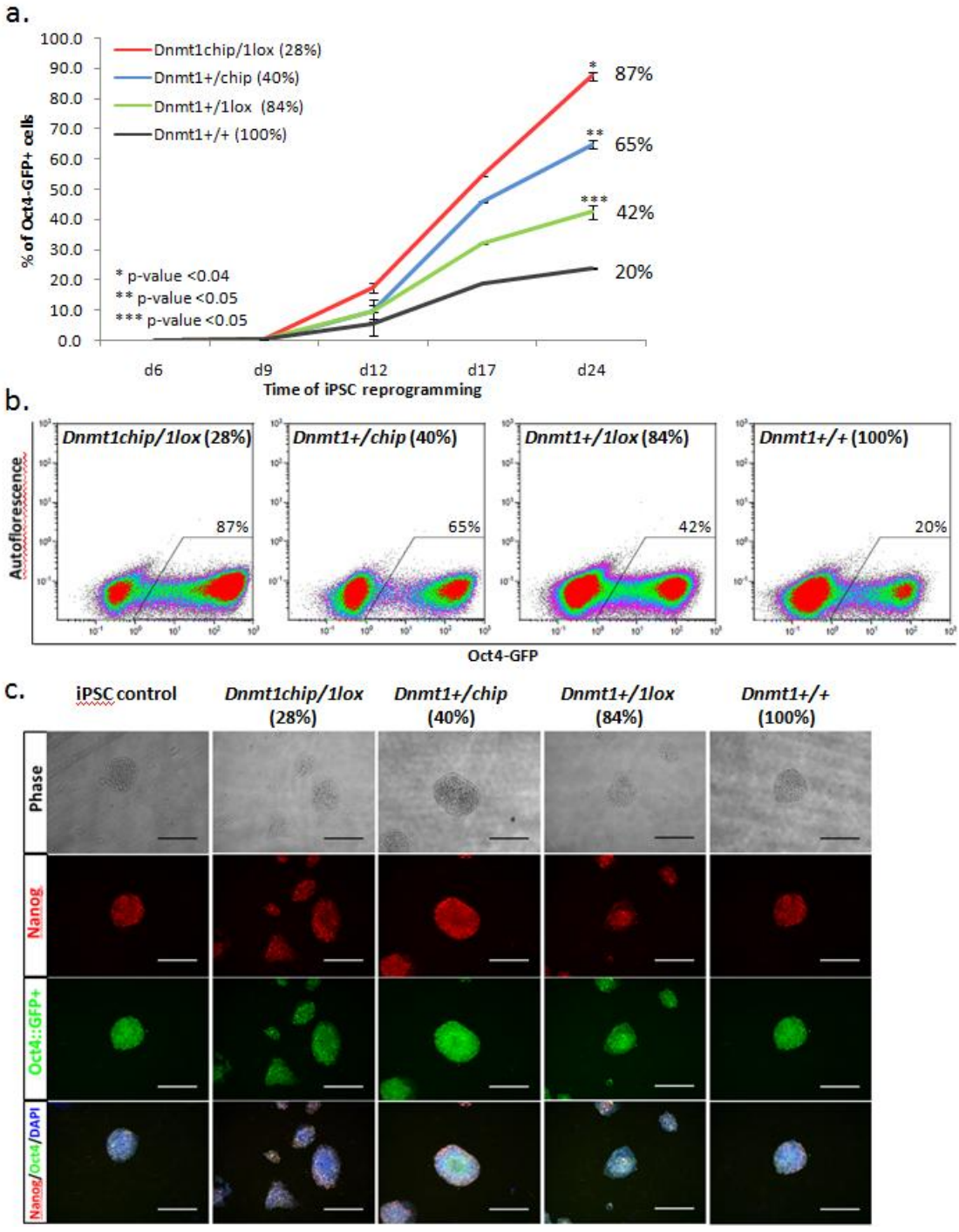
appears to track with the reduced levels of DNMT1 enzyme produced by the different *Dnmt1* expression levels contributes to a corresponding decrease in DNA hypomorphic MEFs (Figure 3.2c). These findings suggest a decrease in endogenous methylation at Oct4 locus, which may affect the efficiency of reprogramming in these hypomorphic *Dnmt1* lines.

Hypomrophic Dnmt1 Enhances the Kinetics and Efficiency of Reprogramming

To see whether hypomorphic $Dnmt1^{chip/1lox}$ MEFs that are the most hypomethylated in the *Oct4* regions can be reprogrammed the fastest and best compared to wild-type control or other cell lines expressing various *Dnmt1* levels, we transduced $Dnmt1^{+/+}$, $Dnmt1^{+/chip}$, $Dnmt1^{+/1lox}$, and $Dnmt1^{chip/1lox}$; *Oct4*::GFP reporter MEFs with four reprogramming factors, *Klf4, Sox2, Oct4,* and *cMyc* and then sorted the number of GFP expressing cells by fluorescent activated cell sorting (FACS). To investigate the kinetics of reprogramming from each cell line, we performed FACS from 6 days to 24 days of reprogramming. Interestingly, we found that the 10%-reduced dosage of *Dnmt1* MEF line, $Dnmt1^{chip/1lox}$, was not only the fastest but also the most efficient facilitator of iPSC reprogramming (Figures 3.3a-b). We discovered that the less the DNMT1 protein level was expressed, the more iPSCs were produced. The 90% reduction in *Dnmt1* level increases the efficiency of reprogramming over 4-fold as compared to the 100% *Dnmt1* expressing wild-type control (Figures 3.3a-b). Also, we found that the $Dnmt1^{+/chip}$ line reprogrammed much faster and more efficiently than the $Dnmt1^{+/1lox}$ line (Figures 3.3a-b), indicating that the lower protein level of Dnmt1 in $Dnmt1^{+/chip}$ line than

Figure 3.3. Dnmt1 hypomethylation promotes the efficiency of MEF reprogramming.
a, Dynamics of the allelic series of *Dnmt1* MEFs iPSC reprogramming. The allelic series of MEFs infected with *Oct4*, *Sox2*, *Klf4*, and *cMyc* were sorted for GFP by using FACS from early (day 6) to late days (day 24) of the reprogramming process. *Dnmt1*$^{chip/1lox}$ line reprogrammed with fastest kinetics and highest efficiency. **b,** FACS analysis, which represent the percentage of Oct4::GFP+ cells from each allelic series of Dnmt1 lines at 24 days of iPSC reprogramming. **c,** NANOG+/Oct4+ iPSC line generated from the allelic series of Dnmt1 MEFs using *Oct4*, *Sox2*, *Klf4*, and *cMyc*, scale bars = 100 μm. For all experiments, error bars represent the standard deviation between three biological replicates and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

Figure 3.3. (Continued)



that in *Dnmt1*$^{+/1lox}$ line aids in iPSC generation. These results suggest that decreased *Dnmt1* levels can significantly enhance the speed and the efficiency of reprogramming.

In addition, to confirm whether another stringent pluripotency marker, Nanog, is activated in our Oct4-GFP positive cells (Silva et al., 2009), we immunostained with the Nanog antibody in each of Oct4 positive reprogrammed cell lines sorted by FACS at 24 days of iPSC reprogramming. We observed that Oct4 positive cells were also Nanog positive, indicating that these sorted cells were iPSCs and were fully reprogrammed at day 24 (Figure 3.3c).

Dnmt1*^{chip} *Allele Transduced Lines Reprogram to Pluripotency by Directly Activating Oct4 and Bypassing the Intermediate States.

To explore the mechanism by which the hypomorphic *Dnmt1*$^{chip/1lox}$ MEFs reprogrammed faster and more efficiently than other cell lines phenotypically, we first hypothesized that the majority of the hypomorphic cell lines are not trapped in the intermediate states that are usually present during the iPSC conversion process in wild-type MEFs, leading to a low efficiency of reprogramming. The activation of stage-specific embryonic antigen-1 (SSEA-1) represents an intermediate step during iPSC reprogramming that precedes the sequential activation of Oct4 and Nanog (Brambrink et al., 2008). Therefore, we can capture reprogramming intermediates by FACS using the marker of SSEA-1 during early iPSC reprogramming process. To validate reducing DNMT1 protein levels generates less intermediates, we first tested with wild-type MEFs, if SSEA-1 positive intermediate populations were indeed enriched for cells that would

form iPSCs. We sorted cells on feeders based on SSEA-1 and Oct4-GFP expression from 6 days to 24 days of reprogramming. Consistent with the previous report (Polo et al., 2012), intermediate cells with the potential to give rise to iPSCs were initially present as a SSEA-1 positive populations, then progressed to Oct4 positive cells, and ultimately transited to the SSEA-1 and Oct4-GFP double positive population (Figure 3.4a).

To verify our hypothesis, we next reprogrammed the allelic series of *Dnmt1* MEFs with four reprogramming factors and sorted these reprogramming cells based on SSEA-1 and Oct4 expressions at day 6, 9, 12, 17, 24 and passage five established iPSC lines from each allelic series of *Dnmt1* MEFs. Surprisingly, the hypomorphic $Dnmt1^{chip/1lox}$ line did not significantly expressed SSEA-1 throughout the entire reprogramming process, but instead it directly activated *Oct4* expression (Figure 3.4a-c). Obviously, the $Dnmt1^{+/chip}$ line behaves differently from wild-type or the $Dnmt1^{+/1lox}$ line, which expresses normal protein levels of DNMT1. Although the $Dnmt1^{+/chip}$ line expressed slightly higher levels of SSEA-1 at later time points of reprogramming (24 days) than the $Dnmt1^{chip/1lox}$ line, it did not activate significant level of SSEA-1, compared to wild-type control or the $Dnmt1^{+/1lox}$ line but expressed relatively low levels of SSEA-1, similar to $Dnmt1^{chip/1lox}$ line throughout the reprogramming process (Figure 3.4a-b).We discovered that both $Dnmt1^{chip}$ alleles introduced cell lines generated less intermediate cells during reprogramming, resulting in fast and efficient iPSC generation. Thus, we concluded that the more we reduced the level of DNMT1 in MEFs, the faster and more efficient the dynamics of iPSCs production.

Figure 3.4. *Dnmt1*^{chip} allele transduced line reprograms to pluripotency by bypassing the intermediate states.
a, FACS analysis of reprogrammable allelic series of Dnmt1 MEFs for SSEA-1 and Oct4 at indicated time points (at day 6, 9, 12, 17, and 24 of reprogramming and in established iPSC lines). Each distribution is indicated in (a) SSEA-1 positive/Oct4 negative, (b) Oct4 positive/SSEA-1 negative, and (c) SSEA-1 positive/Oct4 positive. **b,** Quantification from FACS analysis. Percentage representations of SSEA-1 positive/Oct4 negative cell populations at day 6, 9, 12, 17, and 24 of reprogramming. Percentage of SSEA-1 positive/Oct4 negative cells in *Dnmt1*$^{chip/1lox}$ line (red bars) is extremely low throughout the reprogramming process as compared to wild-type control (black bars) or *Dnmt1*$^{+/1lox}$ cell line (green bars). **c,** Quantification from FACS analysis. Percentage representations of Oct4 positive/SSEA-1 negative cell populations during reprogramming. **d,** Quantification from FACS analysis. Percentage representations of SSEA-1 positive/Oct4 positive cell populations at indicated time points. For all experiments, error bars represent the standard deviation between three biological replicates in three individual experiments, and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

Figure 3.4 (Continue).

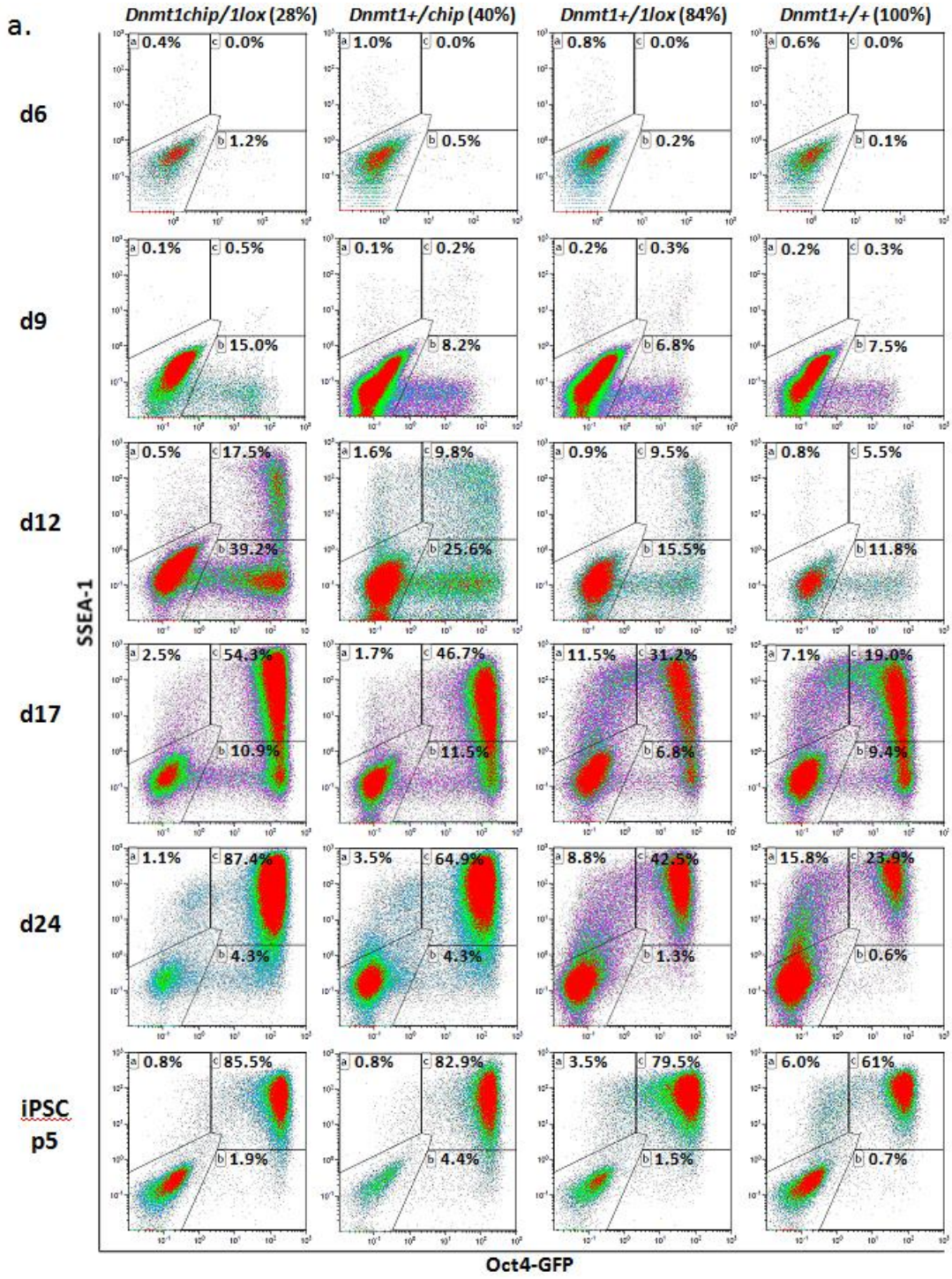
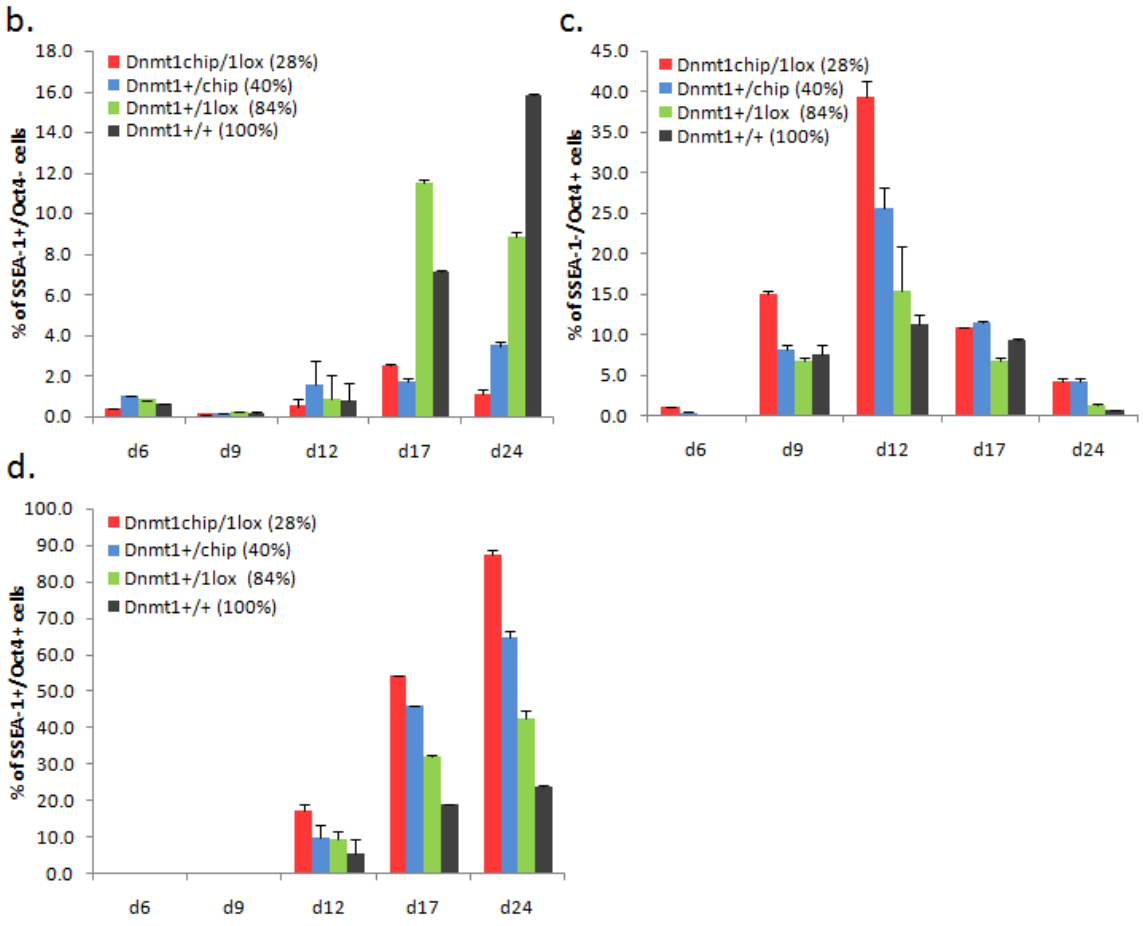


Figure 3.4 (Continue).

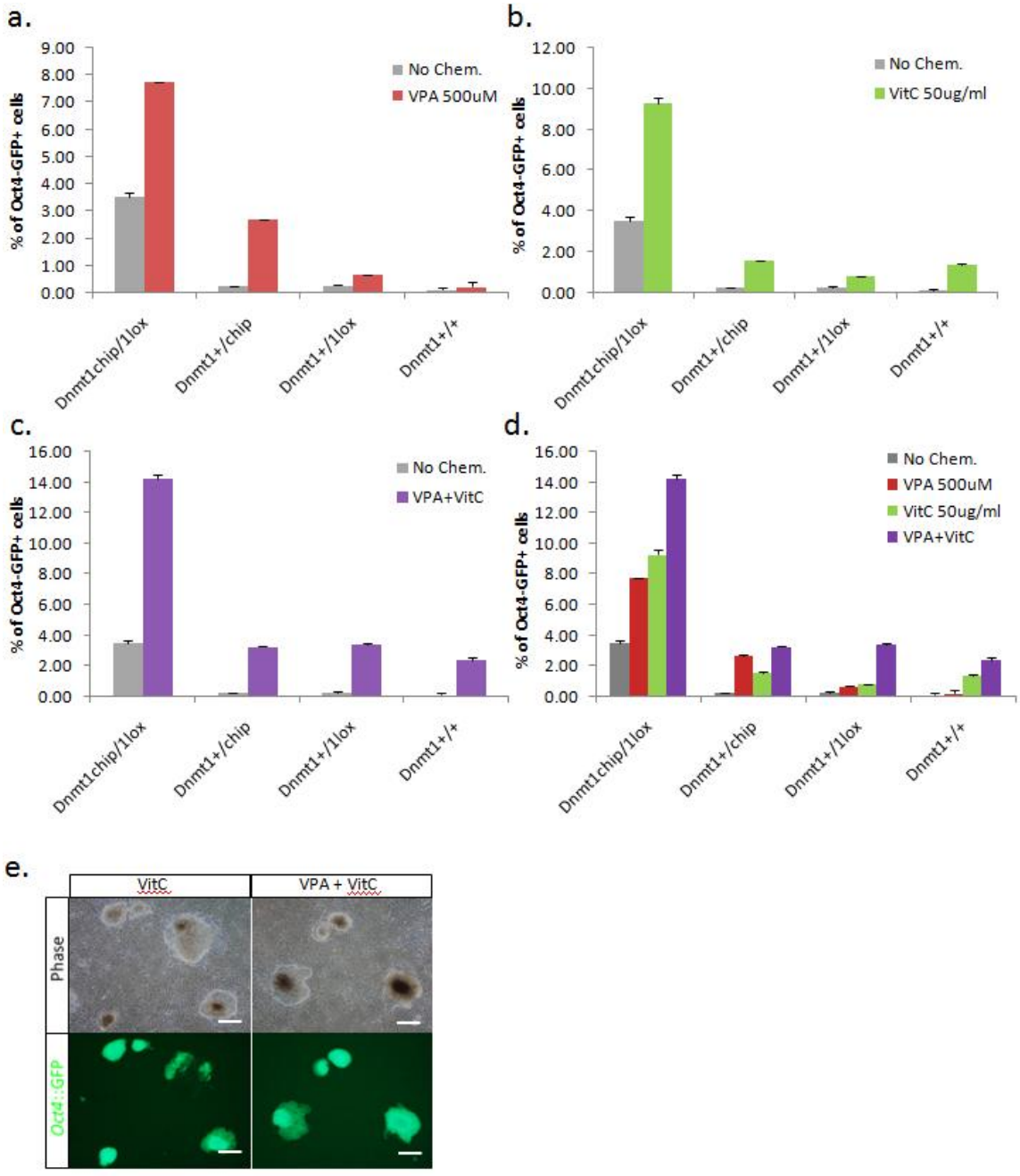


Synergism of DNA Demethylation and Histone Acetylation in Combination with Histone Demethylation Promotes the Efficiency of Reprogramming

High levels of histone acetylation generally correlate with transcriptional activity. Because methylation of DNA recruits histone deacetylases via binding of methylated DNA binding proteins (Csankovszki et al., 2001), it is possible that DNA demethylation and histone acetylation synergistically affect the rate of iPSC reprogramming. To demonstrate how histone acetylation can contribute to iPSC reprogramming synergistically with DNA demethylation, we transduced the allelic series of *Dnmt1 Oct4*::GFP reporter MEFs with the four iPSC reprogramming factors and treated them with an inhibitor of histone deacetylase, Valproic acid (VPA) for 7 days (Huangfu et al, 2008). At 9 days of reprogramming when the VPA treatment was finished, we collected and sorted each cell line for Oct4-GFP positive signal. Consistant to the literature, the addition of 500μM VPA led to a significant increase in the number of resulting GFP positive iPSCs especially for hypomorphic *Dnmt1*$^{chip/1lox}$ line as well as other cell lines including *Dnmt1*$^{+/chip}$ and *Dnmt1*$^{+/1lox}$ line (Figure 3.5a). Strickingly, we found that VPA treatment in *Dnmt1*$^{+/chip}$ line generated over 10-fold more GFP positive cells than the VPA-untreated cell line (Figure 3.5a), indicating that a moderate amount of DNA demethylation can strongly accelerate cells to reach pluripotency by the assistance of an inhibitor of histone deacetylase. These results suggest that histone acethylation can positively synergize with DNA demethylation, thereby generating iPSCs more efficiently (Figure 3.5a).

Figure 3.5. The synergism of DNA demethylation and histone acetylation in combination with histone demethylation promotes the efficiency of reprogramming.
a, Percentage of Oct4::GFP positive cell population in response to VPA quantified by FACS analysis in the allelic series of reprogrammable cells. The allelic series of Dnmt1 MEFs were treated with 500μM VPA for first 7 days after viral *Sox2, Oct4, Klf4* and *cMyc* infections, and then FACS analysis is performed at day 9. **b,** Percentage of Oct4::GFP positive cell population in response to Vitamin C quantified by FACS analysis in the allelic series of reprogrammable cells. 50μg/ml Vitamin C was treated on the four factor infected cells for 7 days, and then FACS analysis is performed at day 9. **c,** Percentage of Oct4::GFP positive cell population in response to VPA and Vitamin C cocktail quantified by FACS analysis in the allelic series of reprogrammable cells. Combination of VPA and vitamin C increases the efficiency of reprogramming the most. **d,** Summary of FACS analysis for Oct4::GFP positive in response to VPA and/or Vitamin C in each cell line. e, P0 mouse iPSC colonies generated from *Dnmt1*$^{+/1lox}$ treated with vitamin C and vitamin C+VPA, respectively, scale bar = 100 μm. For all experiments, error bars represent the standard deviation between three biological replicates in three individual experiments, and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

Figure 3.5 (Continued).



A recent report has shown that histone demethylases enhance somatic cell reprogramming in a vitamin-C-dependent manner (Esteban et al., 2010; Wang et al., 2011). To see whether histone demethylation via vitamin C can synergistically influence the rate of cell conversion process to iPSCs, we added vitamin C to the allelic series of *Dnmt1* cell lines throughout the reprogramming process and sorted Oct4-GFP positive iPSCs by using FACS. Consistant to the literature (Esteban et al., 2010), we could observe that vitamin C accounted for an over 10-fold increased efficiency in reprogramming of wild-type MEFs. Vitamin C significantly enhanced the overall efficiency of reprogramming in all of the allelic series of *Dnmt1* MEFs (Figure 3.5b). When compared to non-treated wild type control, vitamin C-treated hypomorphic $Dnmt1^{chip/1lox}$ line boosted the efficiency of reprogramming over 80-fold in addition to the increase (30-fold) by the effect of DNA demethylation (Figure 3.5b). Moreover, we also found that vitamin C combined with VPA offered a better synergistic effect in iPSC reprogramming. It increased the frequency of reprogramming over 100-fold in both compound treated hypomorphic $Dnmt1^{chip/1lox}$ line than in untreated wild-type control (Figures 3.5d). Therefore, we concluded that inhibition of histone deacetylation and histon demethylation support DNA demethylation, making it easier to overcome roadblocks for iPSC generation that leads to efficient reprogramming.

Reducing Dnmt1 does not Induce Endogenous Oct4 and Nanog Expressions

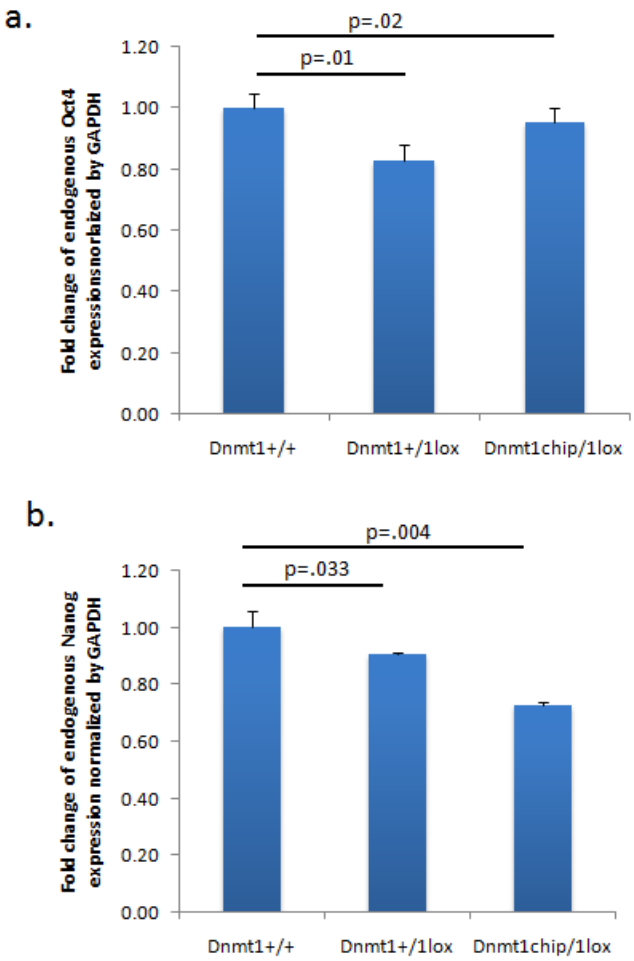
One possible mechanism by which the reduction of Dnmt1 enhances iPSC reprogramming is through the induction of pluripotency gene expressions. To validate

the mechanism that the decreased level of Dnmt1 can highly contribute to facilitate iPSC generation by directly activating their endogenous pluripotency loci, we investigated if any of allelic series of *Dnmt1* MEFs can activate endogenous *Oct4* and *Nanog* expressions by RT-qPCR. However, both *Oct4* and *Nanog* expressions in $Dnmt1^{+/1lox}$ and $Dnmt1^{chip/1lox}$ MEFs were not increased compared to those of wild-type control and were much lower than those of mouse ESC control (data not shown), indicating that *Dnmt1* was not significantly involved in activation of endogenous *Oct4* and *Nanog* gene expressions (Figures 3.6a-b).

Hypomorphic $Dnmt1^{chip/1lox}$ MEF lines have Higher Variations than Other Lines in Reprogramming

To confirm whether individual cell lines which have the same genotype have a similar reprogramming potential to pluripotency, we generated three biological replicates of MEF lines from three separate embryos, wild-type *Dnmt1*, $Dnmt1^{+/chip}$, $Dnmt1^{+/1lox}$, and $Dnmt1^{chip/1lox}$, respectively. The cell lines were transduced with *Klf4*, *Sox2*, *Oct4*, and *cMyc*, and Oct4-GFP positive cells were sorted from 6 to 24 days of reprogramming. Interestingly, hypomorphic $Dnmt1^{chip/1lox}$ MEF lines are more variable than other cell lines in efficiency of reprogramming. Theis data indicate that hypomorphic cell lines are unstable in *in vitro* cell culture systems (Figure 3.3a, black line and 3.5a, grey bars).

Figure 3.6. Reducing *Dnmt1* does not induce endogenous *Oct4* and *Nanog* expression.



a-b, QPCR analysis of endogenous a, Oct4 and b, Nanog on the allelic series of *Dnmt1* MEFs. For all experiments, error bars represent the standard deviation between three biological replicates in three individual experiments, and statistical significance was determined using a two-tailed homoscedastic Student's t-test.

Discussion

In summary, our findings suggest that DNA methylation is a significant impediment to the reprogramming of MEFs to iPSCs. We investigated how quantitatively modulated Dnmt1 levels can contribute to the efficient iPSC reprogramming by generating a transgenic allelic series of *Dnmt1* MEFs that sensitively regulate the levels of *Dnmt1* and by performing FACS analysis with them. We discovered that the sequentially decreasing DNMT1 protein expression generates more iPSCs. Importantly, the 10%-reduced hypomorphic $Dnmt1^{chip/1lox}$ line promotes the rate and efficiency of reprogramming the most compared to wild-type controls, suggesting that highly reduced level of Dnmt1 lowers the kinetic barrier of iPSC reprogramming, thereby facilitating the transition to pluripotency. Consistent with our hypothesis, lowering the level of DNMT1 protein expression allows the reprogramming cells to bypass the intermediate state and directly reach the pluripotent state by activating the *Oct4* gene. Furthermore, DNA demethylation can synergize with histone acethylation and histon demethylation, which is conferred by VPA and vitamin C, respectively, to bolstered the rate of iPSC generation. Therefore, we conclude that reducing Dnmt1 levels removes a critical roadblock of iPSC reprogramming, presumably because the decreased Dnmt1 levels induces global DNA hypomethylations that could easily activate pluripotency genes (Gaudet et al., 2003). Additionally, histone modifications aid in DNA demethylation to demolish the epigenetic barrier of reprogramming.

In this study, we examined the DNA methylation status of ESC marker gene such as Oct4 in all allelic series of *Dnmt1* MEFs. The promoter and enhancer regions of

Oct4 had lower methylation levels in Dnmt1 modulated cell lines, compared to wild-type control. Our results suggest that DNA hypomethylation of *Oct4* in the hypomorphic *Dnmt1* cell line might contribute to facilitate the rate-limiting step of iPSC generation. Further chromatin immunoprecipitation (ChIP)-qPCR analysis to probe the binding of Oct4 along a genomic region of Nanog in the early stage of reprogramming Dnmt1 cell lines could explain how Oct4 DNA binding patterns change as their relative Dnmt1 levels are gradually decreased in the allelic series of *Dnmt1* MEFs. By reduced representation bisulfate sequencing, a recently developed bisulfate-based approach that enriches for genomic regions that contain CpG dinucleotides, thereby reducing the amount of sequencing required for the whole genome assay while capturing the majority of CpG islands and promoters (Meissner et al., 2005), we could analyze and compare differential genomic methylation patterns of somatic genes and pluripotency genes other than *Oct4* in the allelic series of Dnmt1 MEFs. These investigations will provide a clear view of the mechanism by which hypomorphic *Dnmt1*alleles promote reprogramming.

There is still unsolved issue that the $Dnmt1^{+/chip}$ line, which is theoretically 60%reduced levels of the *Dnmt1*gene expression, does not express the expected amount of DNMT1 protein but expresses highly reduced levels (40%) of DNMT1. It is unclear how the hypomorphic allele $Dnmt1^{chip}$, which enhances reprogramming efficiency, affects epigenetic changes in the genome. Therefore, we expect that further characterization of $Dnmt1^{chip}$ gene transduced cell lines by whole genome sequencing will offer critical insights about how DNA demethylation occurred by the hypomorphic

Dnmt1^{chip} allele, and how genome-wide changes of DNA demethylation help facilitate the desired transitions, making reprogramming efficient.

To introduce DNA demethylation into cells, infections with siRNAs or shRNAs against DNA methyltrasferase or blunt treatments with compound DNA methyltransferase inhibitors have commonly been executed. However, they either cause a majority of cell death or induce nonspecific effects. Our study with sensitively modulated Dnmt1 cell lines provides an accurate functional control of DNA methyltransferase. Also, this precise control of *Dnmt1* offers an advanced mechanistic insight into how regulated DNA methyltransferase1 can confer demethylation in DNA and contributes to promote reprogramming. Our findings could provide a foothold to explore the mechanism leading to DNA demethylation in iPSC reprogramming.

Materials and Methods

Derivation of MEFs, Cell Culture, and Genotyping

The allelic series of MEFs were generated using *Dnmt1*$^{2lox/2lox}$ and *Dnmt1*$^{chip/chip}$ mice (Gaudet et al., 2003; Jackson-Grusby et al., 2001). Primary MEFs were isolated at 13.5 days post coitum by decapitation and evisceration of embryos. Only embryos, which were *Oct*::GFP transgene germ-line transmitted in gonad, were selected. Individual carcasses were minced, trypsinized briefly, then disrupted by extensive pipeting. MEFs were cultured in 10% FBS in DMEM medium DNA was extracted from passage 2 MEFs and used for PCR genotyping. The following primer sets were used to distinguish each allelic series of MEFs: Primers for the Dnmt1 5´lox site amplify a 334-bp fragment from the wild-type allele and a 368-bp fragment from the *Dnmt1*^{2lox} allele, respectively. The reverse primer (R2) was added to the previous primer set to distinguish *Dnmt1*^{1lox} allele, which amplify a 280-bp fragment. Primers for *Dnmt1 chip* gene that were designed to detect ampicillin resistent site of Dnmt1 cDNA amplify a 200-bp fragment. *Oct4*::GFP reporter transgene were amplified by the primer sets at 173bp. All animal research was performed under the oversight of the Office of Animal Resources at Harvard University.

Retroviral Infection

For reprogramming experiments, MEFs in passage 2 were infected by retrovirus of four iPS reprogramming factors as previously described using the pMXs vector (Takahashi et al., 2007). MEFs were infected once with concentrated viruses for 20

hours in 10% FBS in DMEM medium, and reprogramming is performed in knockout serum replacement mouse ES medium. The first day that viruses were added was termed "day 1 postinfection." For quantification, *Oct4*::GFP+ colonies were counted at day 15 postinfection unless otherwise stated.

Flow Cytometry and Immunofluorescence

For flow cytometry, harvested cells were incubated with antibodies against SSEA-1 (AlexaFluor647, MC-480, BioLegend) for 20 min. Cells were washed in PBS and then incubated for 30 min. The cells were washed twice in PBS, resuspended in 5% FBS/PBS solution and passed through a 40mm cell strainer to achieve single-cell suspension. Cells were sorted on a FACSAristo (BD Biosciences), Influx cell sorter instrument (BD Biosciences) and/or analyzed in a LSRII (BD Bioscience). All data are synchronized and reanalyzed at Kaluza (Beckman coulter).

Generation of iPS Cells

A P0 colony is generated by FACS for SSEA-1 and GFP double positive cells at 24 days of reprogramming and plated on a feeder layer in mES cell media. When a colony grew bigger, it was incubated in in .25% trypsin (Gibco) for 5 minutes at room temperature before plating on a feeder layer in mES cell media. This process was repeated until passage 5, at which time colonies were trypsinized and passaged in bulk and maintained on feeders in mES cell media.

Antibody Staininig for Nanog

iPS cells were cultured on irradiated MEF feeders in 4 well plates, fixed with 4% paraformaldehyde (PFA) and stained with primary antibodies against mNanog (RCAB001P, ReproCell), followed by staining with the appropriate secondary antibodies conjugated to Alexa Fluor 546 (Invitrogen). Nuclei were counterstained with Hoechst33342 (Sigma).

Western Blots

An anti-Dnmt1 antibody generated in chickens to a carboxy-terminal peptide from mouse Dnmt1 has been described[53]. Passage 2 MEFs were lysed directly in RIPA buffer (Invitrogen) containing protease inhibitors. We detected the Dnmt1 antibody using an anti-chicken IgY-HRP conjugate (Promega) followed by ECL detection (Amersham).

RT-qPCR

RNA was harvested with Trizol (Invitrogen) and purified by RNeasy mini kit (Qiagen) and treated with RNase-free Dnase set (Qiagen) to remove DNA contamination. RNA was reverse transcribed using iScript^{TM}cDNA synthesis kit (Bio-rad). To analyze Dnmt1 expression two sets of primer sequences that amplify exon 4 and exon 12-14 of endogenous Dnmt1 gene, respectively, were used. SYBR green (Bio-rad) was used for QPCR analysis.

Methylation Analyses

Bisulfite conversion of genomic DNA was performed using the Qiagen EpiTect kit according to manufacturer's instructions. Bisulfite converted DNA was amplified with primers covering the Oct4 proximal promoter (PP) and the downstream end of proximal enhancer (3' PE). PCR reactions were performed with MangoTaq DNA Polymerase (Bioline). Cycling conditions were as follows: 2 minutes at 94℃, followed by 34 cycles of denaturation at 94℃ for 45 seconds, annealing at 59°C (PP) or 55°C (3' PE) for 35 seconds, and extension at 72℃ for 30 seconds, followed by a final extension at 72℃ for 5 minutes. PCR products were cloned into pCR™II-TOPO® (Invitrogen) and ~10 clones sequenced per amplicon.

Chromatin Immunoprecipitation and QPCR

Between 5 to 10 × 10^6 cells per single immunoprecipitation were collected, washed with ice-cold PBS, and fixed with 1% formaldehyde. Cross-linked samples were sonicated with a Branson 250 Digital sonifier. Samples were incubated at 4℃ overnight with 1 ug/10^6 cells Oct-3/4 antibody (sc-8628, Santa Cruz). Complexes were precipitated with Protein G Dynabeads (Invitrogen). Beads were washed with low salt immune complex wash, high salt immune complex wash, LiCl immune complex wash, and TE. Chromatin was eluted in elution buffer, and incubated at 65℃ overnight with reverse-crosslinking salt mixture. DNA was purified by standard phenol-chloroform extraction followed by ethanol precipitation. Quantitative PCR reactions were performed using SYBR® Green PCR mix (Applied Biosystems) on an Applied

Biosystems StepOne Plus Real-Time PCR Systems instrument. Fold enrichment was determined by comparing 0.5 ng ChIP-enriched DNA to 0.5 ng whole-cell extract unenriched DNA template.

List of Primers:

Dnmt1_1lox	F 5’ GGGCCAGTTGTGTGACTTGG
	R1 5’ CTTGGGCCTGGATCTTGGGGA
	R2 5’ ATGCATAGGAACAGATGTGTGC
Dnmt1_chip	F 5’ GCTATGTGGCGCGGTATTAT
	R 5’ AAGTTGGCCGCAGTGTTATC
Oct4::GFP transgene	F 5’ AAGTTCATCTGCACCACCG
	R 5’ TCCTTGAAGAAGATGGTGCG
Dnmt1_cDNA_exon4	F 5’ GGAAGGCTACCTGGCTAAAGTCAAG
	R 5’ ACTGAAAGGGTGTCACTGTCCGAC
Dnmt1_cDNA_exon12-14	F 5’ GAGGGAGGAGAAGAGACGAAA
	R 5’ CGGGATCACACTTTTGCTTT
PP	F 5’ ATTTAAGGTAGGGGTGAGAGGATTT
	R 5’ AAATCTAAAACCAAATATCCAACCA
3’ PE	F 5’ GATATGGGTTGAAATATTGGGTTTAT
	R 5’ AATCCTCTCACCCCTACCTTAAAT
Fgf4_control	F 5’ TCACAGAGTCAGGGATGCAC
	R 5’ AGCAGGGGATCTAGGGAACC
Fgf4_OctBinding	F 5’ TCTCCAGGTGACAGTAGCCA
	R 5’ GACTATCCCGCCACCGTTG
Oct4_control	F 5’ TGACTCTTAAAGGGGGCAGA
	R 5’ TTTCTAAGACCCAGGAGGCA
Oct4_OctBinding1	F 5’ GAGTTCTTTCCCAGCCCCTA
	R 5’ ACCTTTTCATGCTGGTGGAC
Oct4_OctBinding2	F 5’ TGAACTGTGGTGGAGAGTGC
	R 5’ CAGAGAAGATGGTTGGGGAG
Oct4_OctBinding3	F 5’ TTGAAAATGAAGGCCTCCTG
	R 5’ CAAGGCTAGAGGGTGGGATT

Acknowledgements

The authors would like to thank A. Into for discussion about the analysis of chip gene integration and primer designs. The authors are grateful for the financial support that made this work possible. K.E. was supported by NIH R01 grant HD045732-03, NIH P01 grant GM099117, the Howard Hughes Medical Institute, and the MacArthur Foundation. A.M was supported by NIH P01 grant GM099117.

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Chapter 4

Conclusions and Promises of Reprogramming Technology

Abstract

Since the birth of induced pluripotent stem cells (iPSCs), rapid progress has been made in dissecting molecular mechanism of defined-factor reprogramming as well as improving the technology. We have controlled genetic and epigenetic functions that are extensively studied in cancer. We have directly modulated Notch signaling pathway, which is the developmental signal of keratinocytes with small molecule signal inhibitors, offering a critical advance in the field of iPSC reprogramming by preserving tumor suppressor activity. In addition, we have generated and reprogrammed with unique cell types that are modulated in the expression of DNMT1, rendering interesting insights for inducing the process of iPSC generation. Using these cell lines as a platform, we can deeply study the molecular mechanism of reprogramming and identify completely chemically defined reprogramming cocktails. Furthermore, transdifferentiation on these cell types may provide multiple routes for producing a desired cell type and improve the efficiency of reprogramming. These new designs could propel these cell conversion technologies forward into therapeutic relevance.

Insights from Keratinocyte reprogramming

Combined Chemical and Keratinocyte Developmental Signal Modulation

We have explored two approaches toward identifying conditions that can replace viral transduction of transcription factors and promote the efficiency of reprogramming. In one, we used small molecules that modulate specific signal transduction pathways of keratinocytes. In the other, we have found that keratinocytes can be reprogrammed with fewer genetic manipulations than previously reported for other somatic cell types.

Various small molecules that have been identified through chemical screening can provide information of their functions in cell signaling (Ichida et al., 2009; Li et al., 2009; Lin et al., 2009), however the precise molecular mechanisms governing iPSC reprogramming are still poorly understood. Our new approach is to directly modulate developmental Notch signals that are highly conserved in the proliferating and differentiating cell type by using a well-known compound inhibitor of Notch signaling, named DAPT. It provided a new insight for understanding the precise molecular events of iPSC generation and new strategies for expanding the translational utility of lineage conversion.

Since Notch signaling is extensively studied with keratinocytes that highly express Notch, we focused our efforts on investigating keratinocyte biology to find a way to control its signaling and convert the cells to the pluripotent state. Notch signaling is known to negatively regulate keratinocyte stem cell (KSC) potential and induce keratinocyte differentiation, by antagonizing p63 expression (Dotto, 2008). Consistently, Notch1 and p21WAF1/Cip1, a ‘canonical’ Notch target in keratinocytes, both suppress

Wnt ligand expression and signaling that also limits stem cell potential (Dotto, 2008). Based on Notch function in keratinocyte differentiation, we hypothesized blocking Notch could induce a less differentiated state in keratinocytes which may increase reprogramming potential and reduce reprogramming factors. A detailed discussion of our findings about unique molecular mechanism of iPSC generation by Notch inhibition will be handled in the following subtopic.

In the end, the inhibition of Notch by DAPT both in mouse and human keratinocytes converts these somatic cells into iPSCs with reduced reprogramming factors, only requiring *Sox2* and *Oct4*. We found that Notch inhibition promotes keratinocyte reprogramming by enhancing long-term proliferation, which implicates the activation of self-renewal. However, more stringent assays are further required to address the question of whether a progenitor population that is prolonged and maintains its self-renewal activity by DAPT treatment is the real cell target which promotes reprogramming. We can sort progenitors and terminally differentiated populations using different cell surface antibodies by FACS (Li et al., 2007) and then reprogram each population to identify which populations can recapitulate keratinocyte reprogramming to pluripotency by Notch inhibition.

Another interesting finding is that a compound inhibitor of Dot1L synergizes with DAPT to enhance the efficiency of reprogramming in *Sox2* and *Oct4*-transduced keratinocytes. This finding suggests that a chromatin modifying compound, in this case, an inhibitor of the histone methyltransferase could add to the effect of inhibiting differentiation to promote reprogramming in keratinocytes.

Our efforts to control normal developmental signals to understand the molecular mechanism of chemical-mediated reprogramming have revealed interactions between signaling pathways during the reprogramming process. Furthermore, our findings with Notch demonstrate that developmental signaling pathways can substantially modulate the activities of reprogramming transcription factors and thereby affect the quality and quantity of the resulting reprogrammed cells.

Oncogenic Factors and p53 Inhibition Dispensable in iPSC Generation

It has been shown previously that activation of the p53 induced p21 pathway during iPSC reprogramming is one of the most significant blockades to generate iPSCs (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009). Ectopic over-expression of reprogramming transcription factors can activate p53, which then induces either up-regulation of p21 or apoptosis, thereby perturbing reprogramming (Hong et al., 2009; Kawamura et al., 2009). Silencing of p53-p21 pathway genes significantly improves the efficiency of reprogramming. This approach became an important pathway to reduce reprogramming factors, because *p53* suppression is necessary to eliminate oncogene *Klf4* and *cMyc* (Kawamura et al., 2009; Okita et al., 2009). However, repressing the tumor suppresser gene *p53* leads to the accumulation of genetic mutation in the resulting iPSCs (Marión et al., 2009). Therefore, our approach in modulating keratinocyte signal pathways to achieve pluripotency became a solution to leave p53 activity intact.

In differentiating keratinocytes, endogenous Notch functions as a direct upstream regulator of p21 expression, and the activated Notch causes growth suppression through upregulated p21 expression (Okuyama et al., 2004). Therefore, Notch inhibition by DAPT treatment following p21 suppression could recover pluripotency from growth arrested cells. In this context, it was a key question whether Notch inhibition by DAPT is independent from p53 down-regulation. Importantly, we have demonstrated that iPSC generation by DAPT in keratinocytes is completely independent from p53 activity as well as apoptosis. Consistent with this result, DAPT did not generate iPSCs with DNA damage. In addition to preserving tumor suppressor activity, the reduction of the anti-proliferative responses of p21 by Notch inhibition allowed keratinocytes follow the reprogramming trajectory to produce iPSCs. Moreover, without *p53* suppression, endogenous gene expression systems of keratinocytes still offer the ability to generate iPSCs without the oncogenic factors *Klf4* and *cMyc*.

Based on the reported signaling pathways in the context of keratinocytes, we have investigated other possible mechanisms of reprogramming, for example, the Wnt pathway. In ESCs, Wnt signaling contributes to the maintenance of pluripotency both in mouse and human (Cai et al., 2007; Ogawa et al., 2006; Sato et al., 2004;Singla et al., 2006). It is also known that Wnt signaling promotes reprogramming of somatic cells to pluripotency (Marson et al., 2008). Fibroblast-derived iPSCs have been generated without exogenous *cMyc*, although the efficiency of reprogramming is reduced. However, this drop in efficiency can be overcome by Wnt/β-catenin stimulation (Marson et al., 2008), due to several possibilities including direct regulation of key endogenous

pluripotency factors such as *Oct4, Sox2,* and *Nanog* by the Wnt pathway, as suggested by genomic studies in ESCs. In the context of keratinocytes, Notch and p21 both suppress Wnt signaling (Devgan et al., 2005).Thus, we could hypothesize that Wnt up-regulation controlled by Notch inhibition using DAPT may contribute significantly to the generation of iPSCs from keratinocytes. However, the majority of the Wnt gene family was down-regulated, when the gene expressions after 1, 2, 4, and 7 days of DAPT treated keratnocytes were investigated. Additionally, when Wnt signaling was activated by treating the cells with the Glycogen Synthase Kinase 3β (GSK3β) inhibitor CHIR99021, we did not observe an increase in the rate of iPSC reprogramming. Therefore, we concluded that Notch inhibition does not stimulate reprogramming by increasing Wnt signaling.

Taken together, our studies demonstrate that pharmacological inhibition of Notch makes it possible to generate iPSCs, rendering *KLF4* and *cMYC* oncogenes dispensable while preserving p53 tumor suppressor activity. Importantly, our findings enabled the production of oncogene-free iPSCs, which could avoid the mutational load.

Insights from Dnmt1 reprogramming

Improving the Efficiency and Kinetics of iPSC reprogramming

The four-factor iPSC reprogramming efficiency from MEFs using retroviral delivery method is roughly 0.001-1%, which is extremely low (Huangfu et al., 2008a and 2008b; Takahashi & Yamanaka, 2006).To complete reprogramming, it required at

least15-20 days. Many efforts have been made to improve the reprogramming efficiency and kinetics by using different methods and protocols, starting with different somatic cell types other than fibroblasts, and treating with chromatin modifying compounds, etc. So far the best increase in the efficiency is 4.4% using four factors in modified mRNA with VPA when derived from fibroblasts, albeit this process still retains slow kinetics (Warren et al., 2010). Therefore, we conclude that the epigenetic barrier to achieve pluripotency is quite huge and difficult to overcome. Based on reported reprogramming processes, we hypothesized DNA methylation may play a critical role in the blockage of reprogramming.

Our approach of reducing the DNMT1 enzyme, which may lead to DNA hypomethylation, increases the efficiency of reprogramming up to six-fold, compared to that of wild-type fibroblast reprogramming. More dramatically, reprogramming in 10%-expressing DNMT1 fibroblasts showed the fastest kinetics by activating the late-pluripotency marker, Oct4 at early time points of reprogramming; iPSCs from this cell line appear the earliest at days 5-6, compared to wild-type iPSCs that appear around 9-10 days. Strickingly, by introducing chromatin modifying compounds, such as VPA and vitamin C, in reduced DNMT1 MEFs, we could increase the efficiency over 100-fold in VPA and vitamin C-treated hypomorphic MEFs as compared to untreated wild-type control. These results suggest that DNA demethylation dramatically synergizes with histone acetylation and histone demethylation, thereby significantly lowering the epigenetic barriers of reprogramming. Therefore, our results have shown that lowering DNMT1 activity has significant contributions in enhancing reprogramming efficiency

and kinetics. Further investigations of how modulated Dnmt1 confers DNA demethylation in genome-wide will provide important information for the exact molecular mechanism and nature of the difficulties in iPSC reprogramming.

Bypassing Partially Reprogrammed Cell States

A possible reason for having low efficiency is that the majority of cells during reprogramming are trapped and cannot overcome the barrier of reprogramming to achieve pluripotency. The intermediate cells appear transiently before converting into iPSCs, but the trapped intermediate cells stably propagate and become partially reprogrammed cells that have completely different characteristics from iPSCs; in partially reprogrammed cell lines, the viral transgenes that are used to deliver reprogramming factors are still activated, while pluripotency genes are silent, showing incomplete demethylation and reactivation (Mikkelson et al., 2008; Takahashi and Yamanaka, 2006).

Transcription factor-induced reprogramming to pluripotency is a gradual process (Brambrink et al., 2008; Polo et al., 2010; Stadtfeld et al., 2008). Based on the analysis of the activation timing of known pluripotency markers during iPSC reprogramming, alkaline phosphates (AP) was first activated, followed by SSEA-1, and then Oct4 and Nanog were observed at later time points of reprogramming in iPSCs that are fully reprogrammed (Brambrink et al., 2008). Consistent with this surface marker analysis, 90-95% cells first lose fibroblast marker Thy1, then progressed to SSEA-1 positive, and ultimately a few cells transited to the SSEA-1 and Oct4 double positive, which

represents iPSCs (Polo et al., 2010). In their further assay, the majority of trapped Thy1 negative and SSEA positive cells are considered the trapped intermediate cells during reprogramming (Polo et al., 2010).

To dissect the mechanism of reprogramming, it could be informative to study reprogramming intermediates. Hochedlinger and colleagues have examined intermediate cell populations poised to becoming iPSCs by genome-wide analyses. To induce pluripotency, two transcriptional waves, first the cMyc/Klf4 wave and second the Oct4/Sox2/Klf4 wave are necessary. Cells that become refractory to obtain pluripotency activate the first wave but fail to initiate the second transcriptional wave. However, only a few cells that pass the second wave and are fully reprogrammed finally obtain changes in DNA methylation.

Looking through the mechanism of reprogramming, our reprogramming study which modulated the final step of iPSC generation, DNA methylation, brought an interesting insight about a roadblock of reprogramming. Our hypomophic *Dnmt1* MEFs did not follow the sequential expression of pluripotency markers during direct reprogramming. MEFs with reduced DNMT1 did not significantly express SSEA-1 throughout the reprogramming process, suggesting that DNA hypomethylation that may be conferred by a low level of *Dnmt1* expression bypassed the formation of intermediate cells. More importantly, they directly activated Oct4 at the early time point of reprogramming, thereby increasing their dynamics.

Further gene expression analysis with the reprogramming intermediates from our allelic series of *Dnmt1* MEFs will provide interesting insights of how reduced DNMT1

cells pass through a different trajectory from the normal reprogramming process, and what is the requiring event to gain pluripotency. This will lead to fundamental advances in our understanding of how reduced levels of *Dnmt1* expression lower the barrier of reprogramming.

Broader Implications of Our Findings from the Cancer Research

The Pleiotropic Functions of Notch in Cancer

Notch signaling is a highly conserved signaling pathway in multicellular organisms. Notch signaling plays a critical role in a wide variety of cellular process, including the maintenance of stem cells, the participation in cell-fate decisions, and the induction of differentiation and proliferation (Leong and Karsan, 2006). Especially, these functions are important in the context of the role of Notch in cancer. The interesting features of Notch in tumorigenesis are its dual functions: oncogenic or tumor repressive depending on the cellular context.

The oncogenic role of Notch was first identified in human T-cell neoplasia. These cancer cells possess a specific chromosomal translocation that attaches a portion of chromosome 7 to chromosome 9, which is characterized by the juxtaposition of the 3' region of the human NOTCH1 gene into the T-cell-receptor-β (TCRβ) locus, resulting in consequent overexpression of the active form of Notch1 (Notch-IC1). Sklar and colleagues first identified the fusion of these two loci in a T-cell acute lymphoblastic leukaemia (T-ALL) (Reynolds et al., 1987). Mice reconstituted with haematopoietic

progenitor cells expressing the human Notch-IC1 proteins develop T-cell leukaemia. They generate immature T cells in the bone marrow (Pear et al., 1996) with simultaneous inhibition of B-cell development, indicating that NOTCH1 signaling drives haematopoietic progenitor cells into the T-cell lineage (Pui et al., 1999).The loss-of-function experiments in which Notch1 in bone-marrow progenitors was inactivated, demonstrated that Notch1 is essential for normal T-cell lineage commitment (Radtke et al., 1999). Aberrant Notch-IC1expression in bone-marrow progenitors leads to immature T cells. Overtime, these mice develop highly aggressive monoclonal T-cell tumors by additional mutations cooperating with the Notch-IC1. This mouse model supported the idea that deregulated expression of the cytoplasmic part of the NOTCH1 protein causes T-ALL in humans. After the discovery of its involvement in T-ALL, Notch signaling was also implicated in various tumors, including breast cancer, medulloblastoma, colorectal cancer, non–small cell lung carcinoma (NSCLC), and melanoma (Ranganathan et al., 2011), although their extent and causality remained to be verified.

Although Notch can contribute to the process of tumorigenesis, it needs to cooperate with other oncoproteins to actually cause cancer. When expressing Notch-IC1 with oncoproteins that commonly override the G1-S checkpoint such as adenovirus E1A (Capobianco et al, 1997), human papillomavirus (HPV) E6 and E7 (Rangarajan et al., 2001), RAS (Fitzgerald et al, 2000), MYC (Girard et al, 1996) or simian virus 40 large T (SV40T) (Bocchetta et al, 2003) *in vitro*, transformation can be induced in various cell types through various mechanisms. NOTCH-IC1 activates PI3K signaling (Rangarajan et al., 2001), which confers resistance to apoptosis (Frisch et al, 1994; Khwaja et al,

1997), expresses ERBB2 (Chen et al, 1997), which stimulates proliferation and growth of cells, and induces NF-κB2 (Oswald et al, 1998), which activates anti-apoptotic proteins (Lin et al, 2003). Therefore, the contribution of Notch to tumorigenesis is unlikely to be the abrogation of the checkpoint, but instead, it might provide complementing oncogenic features, such as a resistance to apoptosis, or differentiation.

In contrast, evidence that components of the same Notch pathway have tumor suppressive functions emerged from studies on skin as well as hematopoietic cells, pancreatic epithelium, and hepatocytes. Tumor suppressor genes are classically defined as genes whose mutation or loss is required for tumor development (Hahn and Weinberg, 2002). The skin is composed of several layers of keratinocytes that are at various stages of differentiation. In normal tissue, proliferating keratinocytes are present mainly in the basal layer of the epithelium and become more differentiated by continuously migrating to upper layer (Rangarajan et al., 2001). In the skin keratinocytes, the tumor suppressive activity of Notch signaling induces cell cycle arrest and differentiation (Lowell et al., 2000; Rangarajan et al., 2001; Nguyen et al., 2006). Conditional deletion of NOTCH1 in keratinocytes results in a significant increase of the basal epidermal layer, largely because of a substantial increase in the number of layers of proliferating cells (Rangarajan et al., 2001). Consistent with a tumor suppressive function for Notch in keratinocytes, NOTCH1 loss of function in mice epithelia develops spontaneous basal cell carcinomas over time (Nicolas et al., 2003).

Various cancers including basal-cell carcinomas are associated with the aberrant Sonic-hedgehog (Shh) signaling and the Wnt signaling. In the presence of Notch

signaling, little or no Shh and Wnt are expressed in normal mouse and human skin. On the contrary, the absence of Notch signaling in mouse or human epidermis results in deregulated Shh signaling, which could lead to the development of basal-cell carcinomas (Thélu et al., 2002). Notch1-deficient mouse skin reactivates Wnt signaling by the increase in β-catenin, which could cooperate with other cellular changes to initiate these cancers (Boonchai et al., 2000; Lo Muzio et al., 2002). These studies suggest that Notch acts as a tumor suppressor.

With respect to iPSC reprogramming, it has been recognized that the extent of cellular differentiation and proliferative capacity of a given somatic cell are important determinants for efficient reprogramming. However, it has not been investigated whether forcing a population of differentiated somatic cells into a more potent "self-renewing" state can increase the reprogramming efficiency. Moreover, the effects of modulating Notch signaling on reprogramming have not been studied in any context. We have used the tumor suppressing function of Notch as one of the dual functions in keratinocytes. By de-repressing growth suppression of Notch using chemicals, we were able to modulate the cell fate and efficiently induce them to the pluripotent state. The γ-secretase inhibitor DAPT or DBZ, which blocks Notch signaling in keratinocytes promote iPSC generation from mouse and human keratinocytes.

To understand the reprogramming mechanism of these γ-secretase inhibitors in iPSC reprogramming, we performed microarray analysis on DAPT-treated reprogramming keratinocytes and searched for significant changes. We found that Wnt signaling genes or Gli2, which is a downstream component of the Shh-signaling were

not activated but the anti-proliferative gene p21 was highly decreased by Notch inhibition during reprogramming. Throughout the confirmation of other signaling pathways involved in Notch signaling during keratinocyte reprogramming, we demonstrated that Notch inhibition by γ-secreatase inhibitors enhances keratinocyte reprogramming by inhibiting p21 activity while preserving another known tumor suppressor p53 expression.

Our critical finding that Notch inhibition renders both oncogenic transgenes and p53 inhibition dispensable during iPSC reprogramming could provide a safer approach for the generation of iPSCs. According to the oncogenic feature of Notch function, inhibited Notch signaling itself during reprogramming can prevent its cooperation with other oncogenes, *Klf4* or *cMyc* from the reprogramming cocktail, although all four factors are present. However, even if the oncogenic function of Notch is reactivated in iPSCs, it wouldn't be cancerous because oncogenic factors from the four factors were omitted by compensating the oncogene expression from the endogenous profile of keratinocytes during reprogramming. When considering the tumor suppressor function of Notch, our findings still offer a safer source of iPSC generation. Although an initial temporal inhibition of Notch is used to induce pluripotency, this would hardly induce mutations, regardless of the presence or absence of Notch signaling in the resulting keratinocyte-derived iPSCs because Notch is under direct control of tumor suppressor p53 in keratinocytes (Lefort et al, 2007; Mandinova et al., 2008). Plus, our finding that p53 expression during the reprogramming process is retained regardless of Notch inhibition, keeps the tumor suppression function maintaining in the cells.

Hypermethylation & Hypomethylation of DNA in Tumor Formation

In normal cells, almost the entire genome is highly methylated except for CpG islands, which are left unmethylated. However, cancer cells are characterized by an abnormal pattern of DNA methylation: DNA hypermethylation at CpG islands and DNA hypomethylation at most non-CpG island regions (Feinberg et al., 1983; Widschwendter et al., 2007). For example, both global DNA hypomethylation and promoter hypermethylation of tumor suppressor genes occur in cancer (Issa, 2000; Ohm et al., 2007). Hypermethylation and hypomethylation of DNA are relative terms and refer to "more" or "less" methylation compared to DNA from healthy cells.

The role of aberrant promoter hypermethylation in the inappropriate silencing of tumor suppressor genes is well documented (Jones and Baylin, 2002). Many studies have confirmed that hypermethylation is predominantly targeted for CpG islands in promoters and first exons of some tumor suppressor genes by de novo methylation (Herman, 1999; Ohm et al., 2007; Schlesinger et al, 2007; Widschwendter et al., 2007). As a consequence, their regulation in cell cycle control, apoptosis, DNA repair, differentiation, cell adhesion, angiogenesis inhibition, and metastasis suppression is stopped (Esteller et al, 2001 and 2006) .Unlike genetic alterations, which lead to immediate disruptions of protein function in tumor, promoter CpG-island hypermethylation is a more gradual and progressive process that leads to a gradual silencing of tumor suppressors (Graff et al., 2000). This represents the characteristics of the heterogeneity of tumor properties. Unlike genetic mutations, epigenetic modifications can be reverted. To reactivate these silenced genes, DNA

methyltransferase inhibitors such as 5-aza-2'-deoxycytidine have been used successfully to treat cancer in humans (Lübbert, 2000).

In addition to the *de novo* methylation, tumor cells are also characterized by a high degree of global DNA hypomethylation. Global loss of DNA methylation generally occurs at centromeric repeats and repetitive sequences in many human cancers. Through the investigation of whether this epigenetic change is a cause or consequence of tumorigenesis, it is currently thought that DNA hypomethylaiton plays a causal role in tumor formation through the enhancement of genomic instability (Ehrlich et al., 2009; Gaudet et al., 2003). Hypomethylation of repeated DNA sequences can disrupt the functions of neighboring genes through transcriptional interference and activate transposable elements that can potentially lead to mutagenesis (Wilson et al., 2007). In addition, this phenomenon has been discovered in the premalignant stages of various types of human cancers, suggesting that hypomethylation may be an early event in carcinogenesis (Ehrlich, 2006). Therefore, global DNA hypomethylation has been used recently as a marker of potential cancer risk (Hsiung et al., 2007; Moore et al., 2008).

In terms of iPSC reprogramming, it has been reported that knockout Dnmt3a and Dnmt3b fibroblasts enable reprogramming to pluripotency with a similar efficiency of wild type cells, suggesting that *de novo* DNA methyltransferase3a and 3b are dispensable for iPSC generation (Pawlak et al., 2011). However, no one has looked at whether knockout Dnmt1 affects iPSC reprogramming, due to the fact that knocking out Dnmt1 leads to embryonic lethality (Li et al., 1992) and is not tolerated in somatic fibroblasts (Jackson-Grusby et al., 2001), indicating the importance of Dnmt1 in

maintaining global DNA methylation patterns for survival of somatic cells. Although it is difficult to investigate changes of reprogramming in response to DNA demethylation by completely knocking-out Dnmt1, we have created a system that sensitively modulates the levels of DNA methylation using the hypomorphic *Dnmt1*^{chip} allele and studied iPSC reprogramming dynamics and efficiency. However, it is unclear how the *Dnmt1*^{chip} allele contributes to the genome at the epigenetic levels.

It has been reported that hypomethylated *Dnmt1*$^{chip/-}$ mice, which reduces Dnmt1 expression to 10% of wild-type levels and results in substantial genome-wide hypomethylation in all tissues, developed aggressive T cell lymphomas that displayed a high frequency of chromosome 15 trisomy at 4 to 8 months of age (Gaudet et al., 2003). Although *Dnmt1*$^{chip/-}$ (or *Dnmt1*$^{chip/1lox}$) mice have the runted phenotype, they are fertile when they are young. However, *Dnmt1*$^{chip/-}$ mice do not breed even with wild-type mice and die after 3 months. It is unclear how the hypomorphic allele, *Dnmt1*^{chip}, affects the epigenetic changes in the genome. Therefore, we can perform whole genome wide bisulfate sequencing with 1 month and 3 months-old T cells and sperm to investigate genome-wide change of DNA methylation.

Potential Opportunities in Reprogramming

Oct4 Screening on Hypomorphic Dnmt1 Cells toward Identifying Transgene-Free Chemical Cocktail

Although key advances toward safer delivery methods of iPSC generation have been achieved with non-integrating gene delivery methods, recombinant proteins, or synthetic mRNAs, a desired approach for the generation of iPSCs with better quality and understanding of the molecular mechanism of reprogramming were demanding. One potential reprogramming method is using chemicals that may provide improved efficiency and specificity in the process of iPSC reprogramming.

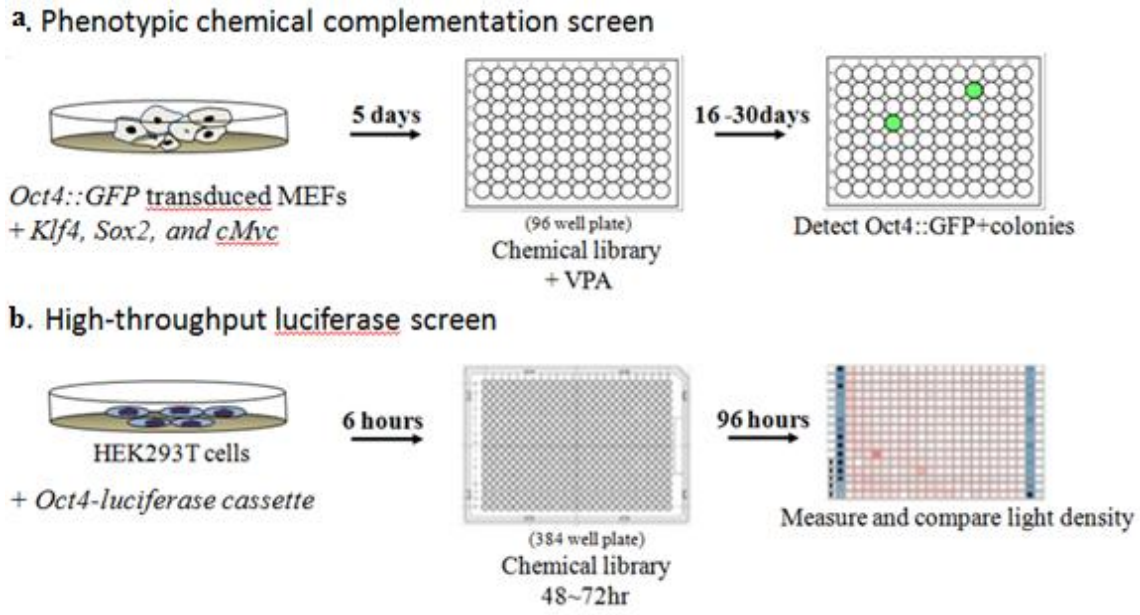
Small molecules have emerged as valuable tools with distinct advantages for iPSC generation because small molecules can identify and manipulate biological pathways that mediate reprogramming. Such compounds would not only facilitate reprogramming but could also be used to characterize the rate-limiting steps (epigenetic mechanisms) in the cell conversion process (Xu et al., 2008). For instance, regulating protein functions is much easier with small molecules rather than by genetic manipulation. Importantly, the effects of small molecules on cells are typically rapid and reversible. We can also fine tune by varying concentrations and combinations of small molecules that could provide temporal and flexible regulation of complex signaling networks. Thus, these characteristics of chemicals could not only offer a chance to understand the molecular mechanism governing iPSC reprogramming but also find a safer alternative to potentially dangerous viral transgenes.

A major advance in iPSC reprogramming is that small molecules that can functionally replace three of the four viral reprogramming factors (i.e., *Sox2, Klf4* and *cMyc*) on MEFs were discovered, thereby enabled the iPSC generation using *Oct4* alone. Controlling direct epigenetic and signaling mechanisms, researcher could find each

transgene replacer, for example, Repsox for *Sox2* replacer (Ichida et al., 2009), and VPA for *Klf4* and *cMyc* replacers on MEFs (Haungfu et al., 2008b). Eventually, a small molecule, a protein arginine methyltransferase inhibitor AMI-5, which enabled reprogramming of MEFs with only the *Oct4* transgene in combination with the transforming growth factor (TGF)-β inhibitor, A-83-01 (Yuan et al., 2011). The small molecule inhibitor of G9a histone methyltransferase, BIX-01294, that replaces the *Oct4* transgene in the presence of the other three factors, *Sox2*, *Klf4* and *c-Myc*, was identified in the reprogramming of neural progenitor cells (NPCs), however, in MEFs this compound cannot substitute for *Oct4*, but *Sox2* (Shi et al., 2008). Since NPCs are not a clinically useful cell source, it is critical to identify a small molecule replacement of *Oct4* on easily accessible cell types, such as patient fibroblasts towards the final goal of a complete chemical cocktail for iPSC generation.

To eliminate the *Oct4* transgene and achieve complete chemical reprogramming, we first screened for an *Oct4* replacer in a variety of ways using a chemical library. Chemicals are able to replace a transgenic factor either by functionally compensating for its absence through other mechanisms or inducing its expression in the somatic genome. We have developed a phenotypic chemical complementation screen and high-throughput luciferase assay in which to fit both mechanisms (Figure 4.1a-b). The systems were applied with a completely annotated chemical library including cell surface receptor agonists, kinase inhibitors, channel modulators, inducers of chromatin modeling and regulators of stem cell properties. We first performed a phenotypic chemical complementation screen for Oct4 replacements by virally transducing *Klf4*, *Sox2*, and

Figure 4.1. Small molecule screening strategies have been applied in identification of *Oct4* chemical replacements.



a, phenotypic chemical complementation screen. Oct4::GFP transduced MEFs were virally tranduced with *Klf4*, *Sox2* and *cMyc*, five days later, were plated into 96-well plates. Each well was administered once every three days with a single compound from the library in the presence of VPA. After 16-30 days, the screen was assayed for GFP positive iPS colonies that had emerged after chemical treatments. **b,** high-throuput luciferase screen. HEK293T cells were transiently transfected with Oct4-luciferease reporter construct, and six hours later, were plated into 384-well plate. Each signal well was treated once every 48 hours a single molecule from the library. After 96hours, the screen was assayed for compounds that induced high luciferase activity.

cMyc in *Oct4*::GFP MEFs in the presence of chromatin modifiers. Secondly, to find direct Oct4 chemical inducers, a high-throughput luciferase screen was executed. Although a few potential Oct4 replacer candidates found in these screens could increase the efficiency of reprogramming under the presence of the *Oct4* transgene, none of the chemicals could replace *Oct4* in the reprogramming assay. Lastly, we hypothesized that *Klf4, Sox2* and *cMyc* transduced partially reprogrammed cells (KSM lines) might easily convert to iPSCs by certain chemical treatments based on several reports that partially reprogrammed cells were able to convert into iPSCs by the treatment of a DNA methyltransferase inhibitor, 5-azacytidine (AZA) (Mikkelson et al., 2008) and that the small molecule RepSox works during the late stages of reprogramming on intermediate cell types which form approximately 10 days after viral transduction (Ichida et al., 2009). Based on this notion, a KSM line was screened by phenotypic chemical complementation using a selectively sorted compound library. However, the KSM line was not responsive to any chemical combination. None of the screening strategy offered any potential *Oct4* chemical replacement. These results indicate that epigenetic barriers to activate pluripotency genes are formidable, therefore, it could be a challenge to re-activate a pluripotent state by controlling signaling pathways using only chemicals.

With respect to mechanistic insights of iPSC generation from NPCs by BIX-01294 that substitutes *Oct4*, this compound may function to facilitate a shift in the epigenetic balance from *Oct4* silencing to activate transcription by histone modifications. Based on the function of BIX-

01294 on NPCs, we can utilize our hyomorphic *Dnmt1* MEFs, which already have significantly lowered epigenetic barriers by reducing DNMT1 levels, in combination with histone modifications as described in previous chapter 3, for the phenotypic screen to identify Oct4 replacements. If this works, we can further explore a transgene-free chemical cocktail by combining various replacement compounds. These findings render a critical insight into the mechanism of iPSC generation. Furthermore, we could apply this protocol to patient specific iPSC generation. Finally, iPSC reprogramming by chemicals would transit a differentiated cell to a pluripotent state through a completely guided direction.

Transdifferentiation on Hypomorphic Dnmt1 Cells

While nuclear reprogramming has revealed the capacity for a differentiated nucleus to be reprogrammed to a pluripotent state, researchers have investigated the potential for directly switching from one mature somatic cell type into another mature somatic cell of alternate fate without reversing into a pluripotent state.

To explore this potential, Blau and colleagues performed experiments in which human amniocytes were fused to mouse muscle cells, resulting in activation of human muscle-specific genes in the heterokaryons within 24 hours (Blau et al., 1983). This suggests that the human fibroblast nuclei have adopted a muscle-specific gene expression program. The prime candidates for converting cell fates are transcription factors, the class of genes that control the expression of other genes.

The initial discoveries in this field have compelled others to identify specific transcription factors for converting from one somatic cell type to another, named transdifferentiation. Taylor and Jones's group first discovered that mouse immortalized (C3H10T1/2) embryonic fibroblasts which do not contain or form myoblasts (Reznikoff et al., 1973) can be readily induced to form functional skeletal myotubes 9 days after the low-dose treatment of AZA, an inhibitor of DNA methylation (Constantinides et al., 1978). This arose from the fusion of mononucleated precursors. In addition, when mouse C3H10T1/2 embryonic fibroblasts were exposed with AZA, they could also observe spontaneous differentiation into adipocytes and chondrocytes (Taylor and Jones, 1979), indicating that DNA methylation restricted gene expression of alternate lineages (Taylor and Jones, 1979). Thereafter, Davis, Weintraub and Lassar sought a gene responsible for the muscle fate switch in AZA-treated fibroblasts by screening a myoblast cDNA library. They reasoned that a small number of powerful muscle inducing genes became de-repressed due to demethylation of DNA. Strickingly, they found that expression of only one transcription factor, MyoD could convert fibroblasts into contracting myoblasts (Davis et al., 1987). This is the first discovery of a transcription factor that can function outside of its normal cellular context to switch cell lineage.

Although AZA causes cytotoxicity to the cells, low dosages (0.03-0.3μM) of exposure for 24 hours made embryonic fibroblasts convert to myotubes, adipocytes and chondrocytes 9-10 days after treatment, when the cells have passed through several division cycles in the absence of AZA (Constantinides et al., 1978; Taylor and Jones, 1979). It will be interesting to first test on our hypomorphic *Dnmt1* MEFs with wild-type

MEF control and C3H10T1/2 cells as a positive control in the presence or absence of AZA to see whether cells that have demethylation of DNA conferred by reduced DNMT1 enzyme themselves can easily convert to myoblasts or any other cell types that have not been reported, and/or they require AZA treatments to transform to myoblasts.

Transdifferentiation from different types of embryonic fibroblasts to myoblasts by MyoD occurred at various efficiency ranges from 3-50%. For example, 53% of transfected C3H10T1/2 colonies were myogenic, whereas 3% of G418 resistant L cells (mouse fibroblasts) and 45% of Swiss 3T3 colonies were myogenic (Davis et al., 1987). This result indicates that MyoD may operate in a permissive environment such that it activates depended on the differentiated state of cells, as if C3H10T1/2 cells are only one step away from myogenic determination. Alternatively, it is conceivable that genes that are essential for myogenic conversion are stably repressed by DNA methylation in certain cell type that has a low efficiency. We could hypothesize that the higher frequency of myogenesis in C3H10T1/2 cells is due to less DNA methylation in myogenesis-controlling genes than Swiss 3T3 cells. Therefore, it will be exciting to explore whether our hypomorphic *Dnmt1* cell line can transdifferentiate to myoblasts by MyoD transfection at higher efficiency than even C3H10T1/2 embryonic fibroblasts. The results will offer insights into the epigenetic barrier, especially DNA methylation, of transdifferentiation between cell lines.

Furthermore, various transdifferentiations from fibroblasts to hepatocyte-like cells, cardiomyocytes, induced neuron (iN), and induced motor neuron (iMN) have been made using defined factors (Ieda et al., 2010; Haung et al., 2011; Sekiya and Suzuki,

2011; Son et al, 2011; Vierbuchen et al., 2010). However, the low efficiency of conversion still remained as a challenge. Thus, we can figure out whether other transdifferentiations can occur at high frequency by our DNA demethylated cell line, delivering a correlation of DNA methylation and lineage barriers.

Closing Remarks

Our findings collectively represent a critical step toward understanding molecular mechanism and epigenetic barriers of iPSC reprogramming. It also provides insights into how genetic and epigenetic modulators of cancer contribute to iPSC derivation. Furthermore, our epigenetically modulated cell line may provide a unique platform in the quest of the reprogramming process. It may allow the generation of iPSCs in completely chemically defined condition without any genetic modification or other cellular conversions efficiently. It is hoped that future application of these types of strategies will further improve the understanding of iPSC generation and will eventually make an impact in cell therapy for human disorders.

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